REFERENCE ONLY

UNIVERSITY OF LONDON THESIS

Degree MD Year 2006 Name of Author McSTAY, M. K. P.

COPYRIGHT
This is a thesis accepted for a Higher Degree of the University of London. It is an unpublished typescript and the copyright is held by the author. All persons consulting the thesis must read and abide by the Copyright Declaration below.

COPYRIGHT DECLARATION
I recognise that the copyright of the above-described thesis rests with the author and that no quotation from it or information derived from it may be published without the prior written consent of the author.

LOANS
Theses may not be lent to individuals, but the Senate House Library may lend a copy to approved libraries within the United Kingdom, for consultation solely on the premises of those libraries. Application should be made to: Inter-Library Loans, Senate House Library, Senate House, Malet Street, London WC1E 7HU.

REPRODUCTION
University of London theses may not be reproduced without explicit written permission from the Senate House Library. Enquiries should be addressed to the Theses Section of the Library. Regulations concerning reproduction vary according to the date of acceptance of the thesis and are listed below as guidelines.

A. Before 1962. Permission granted only upon the prior written consent of the author. (The Senate House Library will provide addresses where possible).

B. 1962 - 1974. In many cases the author has agreed to permit copying upon completion of a Copyright Declaration.

C. 1975 - 1988. Most theses may be copied upon completion of a Copyright Declaration.

D. 1989 onwards. Most theses may be copied.

*This thesis comes within category D.*

☑ This copy has been deposited in the Library of UCL

☐ This copy has been deposited in the Senate House Library, Senate House, Malet Street, London WC1E 7HU.

C:\Documents and Settings\lproctor\Local Settings\Temporary Internet Files\OLK8\Copyright - thesis (2).doc

# Expression of Parathyroid Hormone-Related Protein in Gastrointestinal Malignancies

by

**Mary Kathleen Gemma McStay**

A thesis submitted in fulfilment of the
requirements for the degree of

**Doctor of Medicine**

University College London

University of London

2005

UMI Number: U592299

All rights reserved

INFORMATION TO ALL USERS
The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript
and there are missing pages, these will be noted. Also, if material had to be removed,
a note will indicate the deletion.


UMI U592299
Published by ProQuest LLC 2013. Copyright in the Dissertation held by the Author.
Microform Edition © ProQuest LLC.
All rights reserved. This work is protected against
unauthorized copying under Title 17, United States Code.


ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

## Abstract

Parathyroid hormone-related protein (PTHrP) is a peptide hormone which, when abundantly produced by certain tumours, and released into the systemic circulation, stimulates via an endocrine-like pathway, bone resorption and renal calcium reabsorption, by interacting with a receptor that it shares with parathyroid hormone (PTH), the PTH/PTHrP type 1 receptor (PTH1R). PTHrP is undetectable in the circulation of normal subjects, but is produced in a paracrine/autocrine fashion during foetal and adult life by a number of normal cells and tissues, playing important roles in regulating cell proliferation, differentiation, and development. Tumours derived from the gastrointestinal tract that are not normally associated with hypercalcaemia, such as pancreatic adenocarcinoma, are known to express PTHrP. The expression of PTH1R has not been examined in pancreatic adenocarcinoma: there is no published literature detailing the evaluation of expression of PTHrP and PTH1R in gastrointestinal neuroendocrine tumours or hepatocellular carcinoma (HCC).

PTHrP and PTH1R protein was found, utilising immunohistochemistry, to be expressed by tumour cells in the majority of cases of a series of resection specimens of pancreatic adenocarcinoma and gastrointestinal neuroendocrine tumours, and all HCC studied. Furthermore, PTHrP and PTH1R were detected by western immunoblotting of cell lysates, and by immunohistochemistry in cells, from tumour cell lines derived from the above tumour types. Immunohistochemical expression of PTHrP, PTH1R, and the cell proliferation marker Ki67 was assessed and scored in tissue from normal liver, cirrhotic liver, putative neoplastic precursor lesions [macroregenerative (MRN) and dysplastic nodules (DN)], and HCCs. Immunopositivity for PTHrP correlated with the Ki67 score, and both sequentially increased from normal liver, to cirrhotic liver, to MRNs, to DNs, with a gradient of expression that peaked in tumour cells. Amino-terminal PTHrP (1-34) peptide was fluorescently labelled and observed to be taken up by HepG2 cells, confirming that these cells express functional PTH1R.

The common expression of both PTHrP and PTH1R implies a possible autocrine/paracrine role for PTHrP/PTH1R in these tumours. The positive correlation in expression of PTHrP with Ki67, and sequential rise in the continuum of normal liver through to HCC, qualifies PTHrP as a likely candidate to influence hepatocyte and HCC growth. Agents that target the PTHrP/PTH1R system may have a therapeutic potential in these gastrointestinal malignancies that are frequently refractory to existing treatments.

## Acknowledgements

I would like to thank Dr Martyn Caplin for giving me the opportunity to carry out this research. I thank him for his supervision, encouragement, and corrections. Martyn has instilled in me an interest in the biology of neuroendocrine tumours, and in the management of these patients, that I will have for the rest of my career. He demonstrates a tireless enthusiasm and dedication, not only to his clinical and academic work, but also to his colleagues and family, that I aspire to achieving.

I am indebted to Ms Korsa Khan, Dr Mark Stubbs and Dr Kay Savage for instructing me in the art of immunohistochemistry, western immunoblotting, confocal microscopy, and other techniques employed in this research. I thank them for their patience, and companionship during the many months in the laboratory. Thank you also to Korsa for her friendship, and beautiful singing!

I would like to express my great thanks to Professor Amar Dhillon and Dr Alberto Quaglia, who helped me to select suitable resection specimens and to evaluate the immunohistochemistry. Thank you also to Mr David Bowden and Ms Lynn Murray for their assistance with the figures. I am grateful to Aphton Corporation, Philadelphia, USA for their financial support.

Finally, I would like to thank my parents who have supported me through all of my academic endeavours, and much more. Most importantly I thank Chris for tolerating me while I wrote the thesis, and without whose loving encouragement, it would never have been written. I dedicate this to him.

## Table of Contents

## Figures

## Tables

## Graphs

# Chapter 1 INTRODUCTION

Parathyroid hormone related peptide (PTHrP) was discovered in 1987 as the tumour product responsible for the most common paraneoplastic syndrome, humoral hypercalcaemia of malignancy. When abundantly produced by tumours and released into circulating blood, it stimulates, via an endocrine-like pathway, bone resorption and renal calcium reabsorption, by interacting with a receptor it shares with parathyroid hormone (PTH), called the type 1 PTH receptor (PTH1R). The ability of PTH and PTHrP to bind to a single receptor is explained by limited sequence and conformational homologies within the first thirty-four N-terminal amino acids of the proteins. However, beyond these similarities, PTHrP is distinct from PTH in a number of ways: it is the product of a separate gene; the structure of the rest of the protein is dissimilar to that of PTH; it is a poly-hormone, comprising a family of distinct peptide hormones, arising from post-translational endoproteolytic cleavage of initial PTHrP translational products, each with their own physiological functions; and, in contrast to PTH, PTHrP is a paracrine factor expressed throughout the body at different times in foetal development or adult life. The precise function of PTHrP in most adult tissues has not been completely elucidated, but it has been reported to be important in growth and differentiation as well as cell proliferation and apoptosis.[i] The conservation of the PTHrP gene and amino acid sequences among species suggests that the physiological role of PTHrP is important. This was confirmed by gene-knockout studies that demonstrated that a functional PTHrP gene is required for post-natal survival.[ii]

## 1.1 History

Hypercalcaemia in association with cancer was described in the 1920s, as methods for the measurement for serum calcium were introduced into clinical use. The initial series of patients emphasized the occurrence of hypercalcaemia in the context of skeletal metastases as the underlying cause of the malignancy-associated hypercalcaemia. However, in 1941, Dr. Fuller Albright presented the case of a patient with renal carcinoma, and only one skeletal metastasis, whose hypercalcaemia was

accompanied by hypophosphataemia, which reversed when the tumour was removed. He speculated that PTH, or a related substance, was secreted by the tumour to produce an endocrine or 'humoral' hypercalcaemia.[iii] This observation was re-enforced later by Plimpton and Gelhorn,[iv] reporting the occurrence of hypercalcaemia in a series of patients who had cancer with no apparent evidence of bone metastases.

By 1980, the clinical syndrome of humoral hypercalcaemia of malignancy (HHM) was well-defined biochemically. Patients with HHM were found to resemble patients with primary hyperparathyroidism, not only in that they both displayed hypercalcaemia, but that both groups also displayed elevations in nephrogenous adenosine 3', 5'-cyclic monophosphate (cAMP) excretion, and reductions in renal tubular phosphate reabsorption. In contrast, patients with HHM had reduced circulating PTH, reductions in the activated form of vitamin D, 1, 25-dihydroxyvitamin D, and had higher fractional excretion of calcium. Further, in HHM, the rate of osteoclastic bone absorption is dramatically increased, whilst osteoblastic bone formation is uncoupled and decreased, compared to primary hyperparathyroidism where the rate of osteoclastic bone absorption is increased and coupled to the rate of osteoblastic bone formation. These observations indicated that the humoral factor responsible for the syndrome was not PTH, but a distinct hormone that was capable of interacting with the proximal tubular PTH receptor.

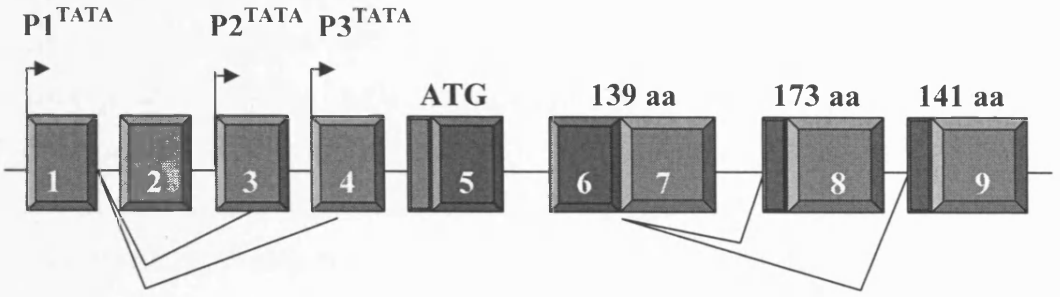
In 1987, using PTH-sensitive renal or osteoblast adenylyl cyclase assays as detection systems, three groups described the purification and N-terminal sequence of PTHrP and the molecular cloning of the corresponding complementary DNA.[v,vi,vii] By the early 1990s, the human,[viii] rat,[ix] mouse[x] and chicken[xi] PTHrP genes had been identified and described in structural terms. Synthetic and recombinant PTHrP had been shown to reproduce the HHM syndrome when infused into animals. In animals with hypercalcaemia caused by allografts of human tumours, the infusion of antibodies to PTHrP was shown to reverse the hypercalcaemia, and elevated circulating PTHrP concentrations were found by radioimmunoassay and immunoradiometric assays in patients with HHM, compared to normal subjects in whom circulating PTHrP is undetectable. This body of evidence established that PTHrP causes hypercalcaemia.[xii, xiii]

It is now widely accepted that PTHrP is the predominant cause of hypercalcaemia in patients with cancer. Among patients with solid tumours and hypercalcaemia, at least 80 percent have increased serum concentrations of PTHrP,[xiv] and up to 20 percent of cancer patients have HHM.[xv, xvi] These patients typically have squamous carcinomas (lung, oesophagus, cervix, head and neck), renal carcinomas, certain lymphomas, bladder carcinoma, and breast adenocarcinoma. When abundantly produced by such tumours and released into the systemic circulation, PTHrP stimulates via an endocrine-like pathway, bone resorption and renal calcium reabsorption, by interacting with the common so-called type 1 PTH receptor, PTH1R.

## 1.2 The PTHrP Gene

The human PTHrP gene is located on the short arm of chromosome 12. It consists of 9 exons and contains at least 3 promoters and a silencer in the 5′- flanking region. The gene for PTH is located on an analogous region on the short arm of chromosome 11. It is clear from the relatedness of the genes that flank the PTH and PTHrP genes, from the sequence homology of the amino-terminal sequences, and from the intron-exon organization of the two genes, that they arose from a common ancestral gene. Through alternate splicing at the 5′- and 3′- ends, the human PTHrP gene yields an array of mRNA species (Figure 1). In particular, alternative 3′- splicing leads to the production of three separate mRNA classes, each encoding a protein with a distinct COOH-terminal. Furthermore, each contains instability motifs (AUUUA motif), involved in the rapid degradation of mRNAs, reminiscent of those found in early response genes which encode cytokines and growth factors.

**Figure 1 Structure of the Human PTHrP gene.**




**Alternate Promoter (P) Usage**

**3' Exons mRNA SpliceVariants**

**Coding Regions**

**Untranslated Regions**

The human PTHrP gene generates an array of mRNA species through alternate splicing at the 5′- and 3′- ends, represented by broken lines below the sequences, generating the production of 3 separate mRNA classes, each encoding PTHrP proteins with separate COOH-terminals. Introns are represented by a line. Promotors are represented by arrows above the sequences.

## 1.3 Regulation of PTHrP mRNA Expression

The physiological importance of alternative splicing of PTHrP remains unknown. Alternative splicing is an important mechanism in the regulation of gene expression, it increases the coding capacity of genes and enhances protein diversity. For example, alternative splicing of the calcitonin gene results in the expression of both calcitonin and calcitonin gene-related protein. These peptides have different functions, the former has a role in calcium haemostasis, the latter is a neurotransmitter and potent

endogenous vasodilator, and both peptides are expressed in a tissue-specific manner.[xvii, xviii] Additionally, alternative splicing may give rise to mRNA variants with different half-lives. For example alternatively spliced insulin-like growth factor-I transcripts within the growth plate have differential mRNA stabilities that may be important in the regulation of chrondocyte growth.[xix]

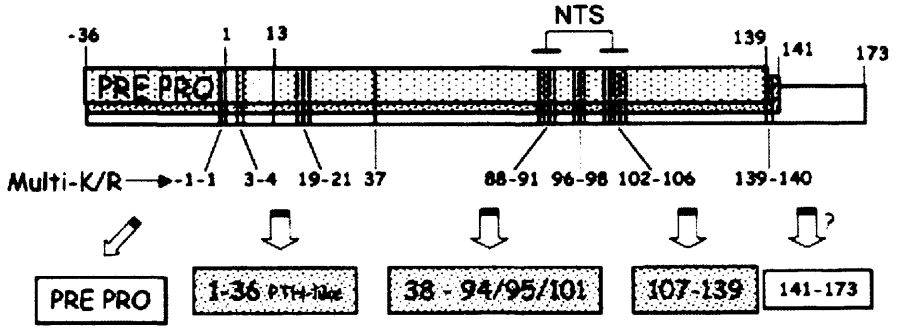
Tissue specific expression of one or more of the alternatively spliced PTHrP mRNA isoforms has been described. A number of groups have demonstrated that all isoforms are expressed in human HaCaT keratinocytes, but that the 139 isoform is predominant, whereas the 141 isoform alone is expressed in human lung tissue.[xx] Evidence of altered regulation of PTHrP mRNA has been demonstrated in different cancer cells, including human lung cancers, which express all PTHrP mRNA isoforms.[xxi] It is known that the expression of PTHrP is induced by a number of signalling molecules, including transforming growth factor-β1 (TGF-β1) and epidermal growth factor (EGF).[xxii, xx] This increased PTHrP mRNA expression has been shown to be due, in part, to the stabilization of PTHrP mRNA, and each isoform has a variable half-life that is differentially altered by cytokines. Hence, in cancer, changes in cell PTHrP mRNA expression may arise through differences in the cytokine milieu or alterations in cell function, which may contribute to the development of HHM or the rate of tumour growth or metastasis.[xxiii] Lung cancers often express higher levels of TGF-β1,[xxiv] which may contribute to changes in PTHrP isoform expression between normal and cancerous tissue.

Increasing evidence indicates that PTHrP mRNA contains at least four CUG codons that have the potential to act as alternative translation sites downstream from the classical AUG start codon. Translation at these CUG sites appears to truncate and disrupt the function of the signal peptide, allowing translated peptide to remain within the cytosol.[xxv] The significance of this will be discussed later.

## 1.4 Translational Products and Post-Translational Processing

Translation of the PTHrP mRNA species give rise by alternate splicing to three related protein isoforms or initial translation products with distinct COOH-terminals. Each of the three isoforms contains a common 36 amino acid putative 'prepro' or signal sequence. Each isoform then contains 139 common amino acids: one terminates at amino acid 139; a second has an additional two amino acids, terminating at position 141; and in humans, a third extends an additional 34 amino acids to terminate at position 173 (Figure 2). Nascent PTHrP isoforms are then processed by members of the prohormone convertase family at multibasic endoproteolytic sites (multi-K/R) to at least three fragments: N-terminal PTHrP (1-36); a mid-region PTHrP (38-64); and, a C-terminal PTHrP (107-139).[xxvi] In humans, a further C-terminal species, PTHrP (141-173) is produced. The precise amino acid sequence of this fragment is unknown. The function of the signal peptide is to direct PTHrP, following translation on ribosomes, to the endoplasmic reticulum.[xxv]

**Figure 2 Structure of the three initial PTHrP isoforms.**




This figure shows the three initial PTHrP isoforms arising from alternative splicing, posttranslational processing at multibasic endoproteolytic sites (multi-K/R), and the amino-terminal, mid-region and carboxy-terminal mature secretory forms of PTHrP, as they are understood at present. PTHrP (1-36) is the mature PTHrP species exhibiting PTH-like properties, not only in bone and kidney, but also in a number of other systems, including the cardiovascular system. This region contains the 1 – 13 region in which 8 aminoacid are homologous to the analogous region in PTH. Note also the bipartite nuclear/nucleolar targeted sequence (NTS, also referred to as "NLS") in the 88 – 106 region. From Clemens et al, 2001.[i]

The N-terminal PTHrP (1-36) is structurally related to PTH. The region of sequence homology between the two peptides runs from amino acid 1 to amino acid 13. Although amino acids 14-34 of PTH and PTHrP share no primary sequence homology, these regions are highly similar in conformational terms, and compete with similar affinity to the PTH1R.

The 35-111 amino acid region is conserved among the four species for which the sequence is known (human, mouse, rat, and chicken), with the human and rat sequence differing by only two amino acids. This region contains a basic bipartite nuclear/nucleolar localization sequence (NLS) in its 88-107 region similar to the NLS in viral and mammalian transcription factors.

From amino acids 111-139, there is some degree of sequence conservation among species. The 141-173 region, which is derived from exon 5 of the human gene is not however homologous with any other peptide.[xxvii] This exon is not present in the rodent and chicken PTHrP gene. Therefore this region of the peptide appears to be human specific. In a recent study, a second tetrabasic KKKK (147-150) motif has been proven to determine nuclear/nucleolar localisation of PTHrP (1-173) in human chondrocytes.[xxviii]

## 1.5 Mature Secretory Peptides and Methods of Secretion

PTHrP isoforms may be secreted as intact peptides, or be processed into several mature secretory peptides. Processed secretory forms of peptide predominate over intact unprocessed peptide.[xxix] Each peptide has its own biological properties and probably acts through distinct receptors.

One mature secretory form of PTHrP is PTHrP (1-36), which binds to the only characterised PTHrP receptor, the PTH1R. A midregion form of PTHrP that begins at amino acid 38 and has a COOH terminal at amino acid 94 has been identified, and a third peptide from amino acid 107 also exists. This latter form of PTHrP has been named 'osteostatin', to indicate the potency of synthetic PTHrP (107-139) as an inhibitor of osteoclastic bone resorption.[xxx] Work from several laboratories has identified a peptide recognised by antisera directed against this region of the peptide

within cells, in conditioned medium, and in circulating serum from humans,[xxxi] however the precise initial and final amino acid of the authentic form of osteostatin has not yet been identified.

Immunoassays against the human 140-173 sequence of PTHrP have also demonstrated the presence of a peptide with this immunoreactivity in plasma, tissue, and conditioned medium from normal cells and from cells transfected with PTHrP (1-173) cDNA. Again the precise origin and terminal amino acids of this peptide are unknown, and its physiological function is entirely speculative at present.

Other secretory peptide forms of PTHrP exist, which undergo further post-translational modification by glycosylation. Keratinocytes have been shown to secrete a heavily glycosylated form of the peptide that begins at the first amino acid (Ala-1), and extends for about 80 amino acids. The mass of the core protein is about 10 000 kDa, whilst the mass of the glycosylated protein is approximately 18 000 kDa.[xxxii] The glycosylation is presumed to be O-glycosylation, since the PTHrP sequence contains no N-glycosylation consensus sequences, and is probably in the midregion of the peptide, since this region is rich in threonines and serines. The expression and release of a glycosylated form of full length PTHrP has also been found in coronary endothelial cells.[xxxiii]

PTHrP is unusual in that it is both a neuroendocrine peptide and a growth factor or cytokine. It is the product of a broad variety of neuroendocrine cell types (e.g. pancreatic islet cells, adrenal medullary cells), in which it is packaged into secretory granules, and undergoes extensive post-translational processing in a manner analogous to chromogranin A and somatostatin. However, like a cytokine or growth factor, PTHrP is processed and secreted in a constitutive fashion from cells which do not contain neuroendocrine secretory machinery such as vascular smooth muscle cells, osteoblasts and keratinocytes.[xxxiv] This duality of secretory mechanisms has not been described for other peptides.

## 1.6 PTHrP Receptors

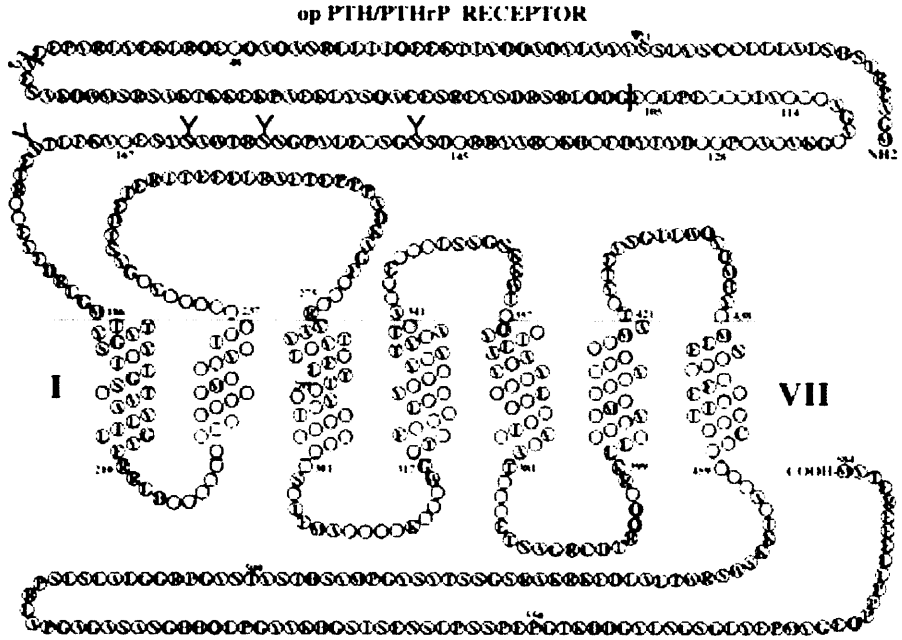
### 1.6.a The Type 1 PTH Receptor

The type 1 PTH Receptor, so called PTH1R, recognizes equally PTH (1-34) and PTHrP (1-36) and is expressed at high levels in bone and kidney, in which it mediates the classical effects of PTH and PTHrP, stimulating bone resorption and renal calcium reabsorption. PTH1R is also expressed at lower levels in a number of tissues in which it mediates a large array of non-traditional paracrine and autocrine functions and responds to locally produced PTHrP.

#### *1.6.a.1 Receptor Structure*

PTH1R is the only PTHrP receptor that has been characterized. It has been cloned in rat,[xxxv] mouse,[xxxvi,] opossum,[xxxvii] and human, [xxxviii] and is a G protein-coupled receptor (GPCR) with 7 membrane-spanning helixes (Figure 3).[xxxix] GPCRs are the largest family of integral membrane receptors, and mediate the action of numerous hormones, cytokines, sensory, and chemical signals. These receptors are classified in three major groups based on sequence homologies.[xl] PTH1R is a member of class B (Class II), which consists of peptide hormone and neuropeptide GPCRs, including receptors for secretin, calcitonin, and glucagon. The PTH1R receptor gene has been localised to human chromosome 3, and has a complex organisation with 14 exons encoding the putative receptor protein.[xli] Three 5′ non-translated exons lie upstream from the signal peptide of the classical receptor. By using alternative transcription start sites in a tissue-specific manner, four alternatively spliced transcripts are transcribed with unique tissue distribution.[xlii]

**Figure 3 Amino Acid Sequence and Predicted Topological Structure of Opossum PTH1R.**




***Key**: Arrow*, Predicted cleavage site of the signal peptide. *Gray circles*, Conserved residues with the rat secretin receptor. *Y,* theoretical sites of N-glycosylation. [ ], boundaries of exon 2. From Vilardaga et al, 2001.[i]

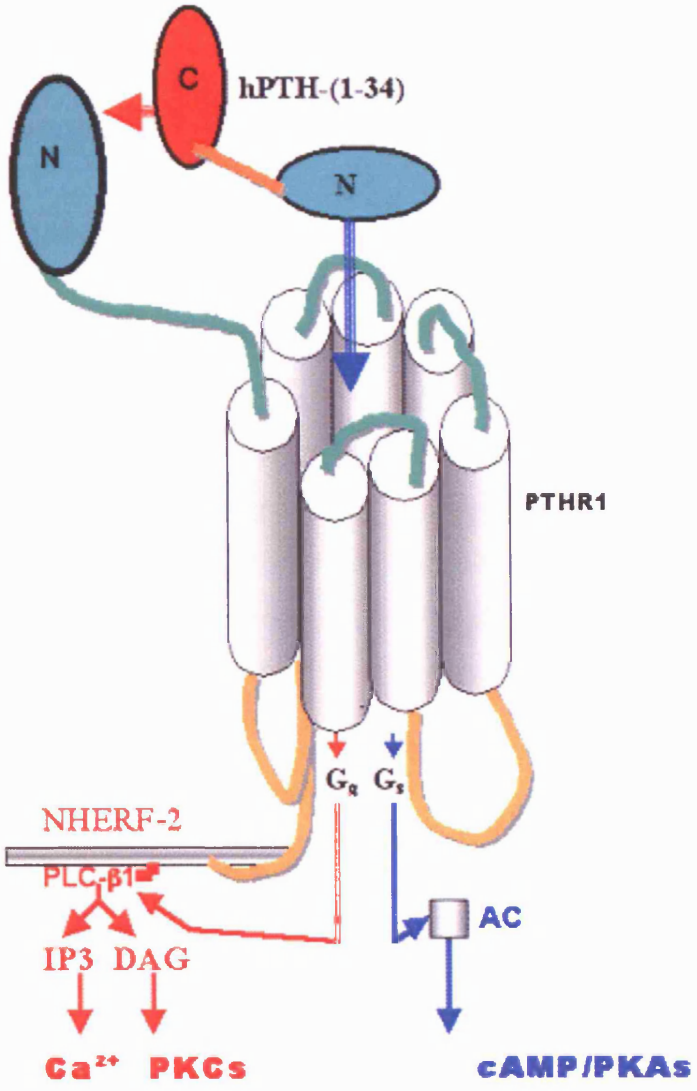
*1.6.a.2 PTH1R Activation and Signalling*

PTH (1-34) or PTHrP (1-36) bind to PTH1R and signal through both adenylyl cyclase (AC) and phospholipase C (PLC). The significant sequence homology in the first thirteen amino acid residues of the two peptides reflects the importance of the N-terminal residues in PTH1R signalling. Between PTH and PTHrP, sequence homology decreases markedly in the 14-34 region, where only three amino acids are identical. For both PTH and PTHrP, the 15-34 region functions as the principal

PTH1R binding domain, by interacting with the extracellular N-terminal domain of the PTH1R. These portions of the two peptides probably interact with the same or overlapping binding sites on the receptor, as the two fragments compete equally for binding with radiolabelled PTH (1-34) or PTHrP (1-36) to the PTH1R.[xliv, xlv] These data also suggest that the two divergent receptor-binding domains of PTH and PTHrP adopt similar conformations.

The early N-terminal portions of each peptide contacts with the extracellular loops of transmembrane helixes 5 and 6 of the receptor, and this interaction is essential for AC stimulation. Adenylyl cyclase activation is thought to be responsible for most cellular responses to PTH and PTHrP. This pathway has been widely studied in a variety of cellular settings and typically elicits a robust and sensitive response to agonist ligands. In comparison, agonist efficacy and potency profiles observed in assays of the PLC pathway are generally lower.[xlvi] Phospholipase C seems to be selectively activated via binding of the PTH1R's intracellular C-terminal tail to the PDZ-2 domain of Na(+)/H(+) exchanger regulatory factor (NHERF) 2 (Figure 4). PTH treatment of cells that express the NHERF2-PTH1R complex markedly activates PLC and inhibits AC through stimulation of inhibitory G proteins (G(i/o) proteins).[xlvii] Thus, it seems that NHERF-2 is a 'switch' that selects the signal pathways used by the activated PTH1R.[xlviii] This may explain why PTH fragments with disabled or truncated N-noses, such as 1-desamino-PTH(1-34), PTH-(3-34), and PTH-(13-34), can in some cells still strongly stimulate PLC and activate protein kinase C but not AC.[xlix] It may also be that the ability of these N-terminally truncated PTH fragments to activate the PLC/$Ca^{2+}$/PKCs mechanism in some cells but not others is a function of the availability of NHERF-2 adaptors.

**Figure 4** **Illustration of PTH (1-34) binding and signalling to PTH1R.**




In this figure phospholipase C (PLC) is selectively activated via binding of the PTH1R's intracellular C-terminal tail to Na(+)/H(+) exchanger regulatory factor (NHERF) 2. Adenylyl cyclase (AC) is inhibited through stimulation of inhibitory G proteins.

*1.6.a.3 Desentisization and Internalization*

The magnitude of hormone-induced physiological responses is normally tightly linked to the balance between GPCR signal generation and signal termination. The GPCR is activated by occupation which leads to biochemical events that uncouple the receptor from its cognate G-protein, producing a non-signalling, desensitized receptor. This is generally accompanied or followed by internalization, and depending on the particular receptor and cell, resensitization and receptor recycling to the cell membrane.[l]

Vilardaga et al reported the results of experiments using model HEK293 mammalian cells engineered to express human PTH1R.[xliii] The investigators showed that signalling by the PTH-PTH1R complex stops when the complex is endocytosed, which is rapid and occurs with a half-time of 3-5 minutes. Then, with a half-time of 2-4 hours, the PTH1R slowly reappears on the cell surface, ready to work again. This recycling requires a specific "recycling domain," which is located more distally than the "endocytosis domain" in the receptor's C-tail and is activated by the association of the phosphorylated receptor with the beta-arrestin-2 scaffolding protein. The PTH-PTH1R-beta-arrestin-2 complex is then targeted to a clathrin pit and carried into the cytoplasm in an endocytic vesicle. PTH is removed, PTH1R is dephosphorylated, functionally resensitized, and then put back on the cell surface, leaving the beta-arrestin-2 behind in the cytoplasm. The C-terminal tail of the receptor is phosphorylated by either second messenger-dependent kinases (AC/PLC) or by G-protein-coupled receptor kinase-2 (GRK2). Phosphorylation alone is insufficient to induce PTH1R internalization, but may produce a local concentration of negative charges that promotes binding to the positively charged recognition domain of an arrestin, thereby stabilizing the formation of the receptor-arrestin complex.[li]

PTH1R appears to exhibit ligand-specific inactivation and internalization, just as it exhibits ligand-specific activation. Sneddon et al using kidney distal convoluted tubule cells transfected with human PTH1R/enhanced green fluorescent protein fusion protein, employed quantitative, real-time fluorescence microscopy to analyse receptor internalization. In these cells, PTH (1-34) activated AC and PLC, and PTH1R endocytosis. Using PTHrP peptides truncated at the N or C terminus, they found that progressively N-truncated PTH peptides internalized the PTH1R without concomitant

receptor activation. Conversely, a C-truncated PTH peptide efficiently activated the PTH1R but did not induce internalization.[lii] Additionally, this study provided compelling evidence that GCPR activation and receptor endocytosis can be dissociated. This dissociation is likely to depend upon different intracellular conformations of the PTH1R. Alternatively phosphorylation of the C-terminal tail may induce a conformational change in arrestin that permits high-affinity interaction between the two proteins.

*1.6.a.4 PTH1R Nuclear Localisation*

Immunoreactive PTH1R has been demonstrated within the nucleus of various tissues in a broad range of cell types.[liii] Watson et al identified a nuclear localization sequence (NLS) between residues 446 and 473. By transfecting LLC-PK1 porcine kidney cells, which do not normally express PTH1R, with a vector containing the human wild-type PTH1R gene, they demonstrated that the transgene product got into the nucleus. When they transfected a vector containing a gene encoding a PTH1R without the NLS, however, the transgene product could only collect in a perinuclear ring. Thus, it seems that PTH1R has the ability to go directly to the nucleus instead of to the cell surface, and that PTHrP might act on nuclear function through binding to intranuclear PTH1R. Alternatively PTHrP may be internalized via receptor-mediated endocytosis, and be subsequently directed to the nucleus.

### 1.6.a Other Receptors for PTHrP (1-36)

There is some evidence for the existence of a novel receptor for N-terminal PTHrP in keratinocytes, insulinoma cells, lymphocytes, and squamous cell carcinoma cells.[liv,lv] Both PTHrP (1-36) and PTH (1-34) have been proven to produce large increases in intracellular free calcium in keratinocytes and squamous cell carcinoma cell lines, but no increases in cyclic AMP, suggesting the existence of an alternate receptor for N-terminal PTH or PTHrP. Such a receptor has however not been conclusively identified.

Evidence for an additional receptor specific for PTHrP (1-36) has been observed in the rat supraoptic nucleus, in which it mediates a cyclic AMP-dependent stimulatory

effect, on vasopressin release.[lvi] In these studies, PTH(1-34) was ineffective or unable to bind to the nucleus or displace bound PTHrP. Additionally a receptor that selectively binds PTHrP(1-36) and stimulates cyclic AMP has been identified in zebrafish.[lvii,lviii] Whether a mammalian homologue of this subtype of receptor exists, remains to be determined.

### 1.6.c Receptors for Mid-regional and C-terminal PTHrP Species

The pro-hormone nature of PTHrP, with the documented existence of mid-region and carboxy-terminal secretory forms, strongly suggests the existence of additional receptors recognizing these mid region and C-terminal PTHrP peptides, as these peptides do not interact with the PTH1R and have no PTH-like activities. These receptors have not yet been identified.

## 1.7 An Intracrine Pathway for PTHrP

In addition to the classical autocrine/paracrine roles of PTHrP, for the most part, by binding via its N-terminal region [PTHrP(1-34)] to PTH1R, PTHrP has been observed to have intracrine actions, entering the cell nucleus under the direction of a nuclear localisation signal (NLS). The latter concept first arose from the observation that the PTHrP sequence contains a NLS composed of multibasic amino acids in the 88-106 region of the peptide, analogous to the prototypical nucleoplasmin NLS.[lix] This sequence also bears homology to sequences in human retroviruses shown to target regulatory proteins to the nucleus. A number of groups have shown that this sequence is necessary and sufficient for the nuclear localisation of PTHrP.[lx, lxi] The mechanisms regarding the access, regulation and consequences of nuclear entry of PTHrP are areas of active research.

### 1.7.a Mechanisms Underlying Translocation of PTHrP to the Nucleus

How does PTHrP, which undergoes extensive post-translational processing before secretion, enter the nucleus from the cytoplasm? Three possible mechanisms exist: 1) it is secreted and then re-enters the cell cytoplasm by endocytosis; 2) it does not enter the endoplasmic reticulum, but remains in the cytoplasm where it directly accesses the nuclear core; or, 3) it travels retrograde from the endoplasmic reticulum, to the cytoplasm, and then enters the nucleus (Figure 5). Evidence exists to support each of these possible mechanisms.

#### *1.7.a.1 Secretion and Endocytosis*

The mechanism by which a secretory protein may gain access to the cytoplasm after secretion may involve endocytosis-dependent pathways. There are numerous examples of this mechanism for other peptide hormones, and cytokines, including angiogenin, angiotensin, and platelet-derived growth factor. Although endocytosis of PTH1R, with subsequent PTHrP transport and localisation to the nucleus has been demonstrated,[lxii,lxiii] other studies do not support this concept.[lxiv]

It is possible that a receptor distinct from the PTH1R mediates PTHrP endocytosis. Aarts et al demonstrated that full-length PTHrP (1-141), secreted from COS-1 kidney fibroblast cells transfected with PTHrP, can be endocytosed and subsequently targeted to the nucleus. This endocytosis was NLS-dependent, but did not require the PTH1R.[lxv] However, full-length PTHrP, which is the only secreted PTHrP species that contains the NLS, is a minor secretory form of PTHrP, making this pathway to the nucleus for PTHrP less likely. Alternatively, endocytosed PTHrP could reach the nucleus by binding to an intracellular form of the PTH1R. Joun et al described a novel splice variant of the PTH1R, which preferentially localized to the cytoplasm.[xlii] Such a pathway has also been described for fibroblast growth factor 2 (FGF-2).[lxvi]

#### *1.7.a.2 Alternative Translational Start Sites*

As previously described, PTHrP mRNA contains at least four CUG codons within the signal peptide region that have the potential to serve as alternative translational start sites.[lxvii,lxviii] Initiation of translation at these start sites appears to truncate the signal peptide, and disrupt its function. Thus the nascently translated PTHrP avoids entry

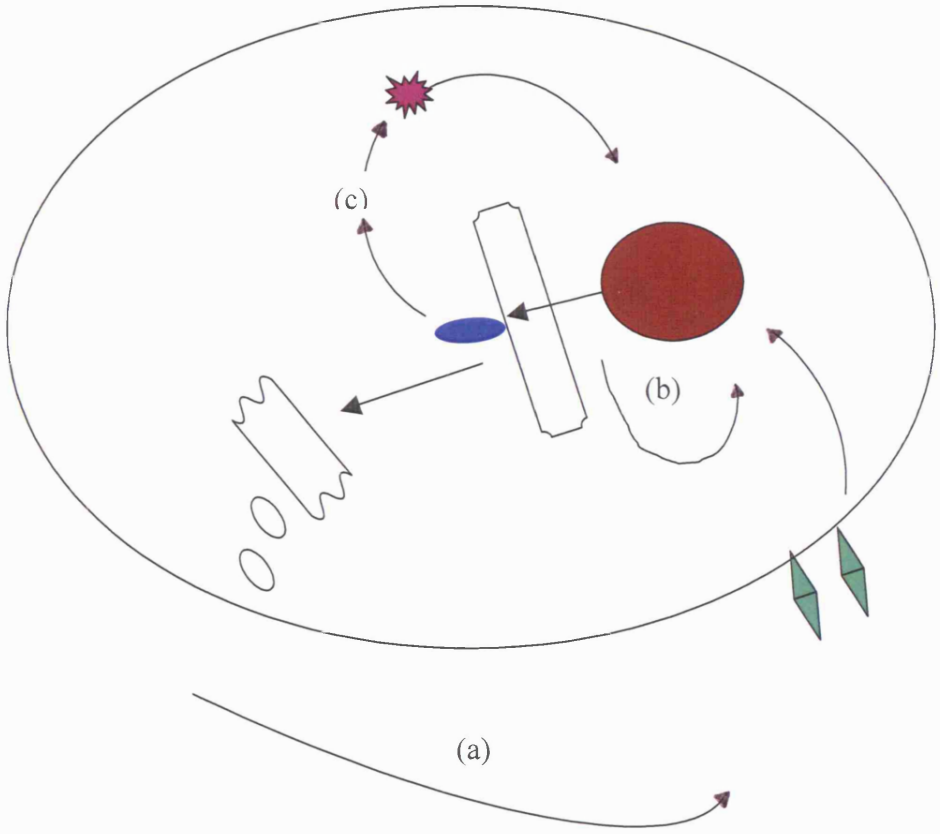
into the endoplasmic reticulum, and remains in the cytoplasm where it is free to travel to the nucleus under the direction of the NLS. This scenario, which involves alternative initiation start site mechanisms at non-AUG codons, has been documented for FGF-2, where alternative initiation of FGF-2 mRNA occurs at three downstream CUG codons in addition to the conventional AUG start codon.[lxix] Therefore alternative initiation of translation results not only in the synthesis of the AUG-initiated form of FGF-2 that enters the endoplasmic reticulum, but also of three CUG –initiated FGF variants that remain in the nucleus and translocate to the nucleus, where they have a variety of biological effects.

*1.7.a.3 Retrograde Trafficking from Endoplasmic Reticulum to the Nucleus*

Recent evidence suggests that secreted proteins can reverse-translocate from the endoplasmic reticulum to the cytoplasm. Misfolded proteins that would classically undergo degradation in the endoplasmic reticulum, can be retrogradely translocated from the endoplasmic reticulum to the cytoplasm, and then degraded by cytoplasmic ubiquitin-proteosomes.[lxx] PTHrP is a secretory protein that undergoes proteolysis through the ubiquitin-proteosome pathway,[lxxi,lxxii]and so it is possible that PTHrP can be translocated back to the cytoplasm by specific endoplasmic reticulum sequestering proteins, and from there gain access to the nucleus without undergoing proteasomal degradation. The observation that the expression of a PTHrP construct that contains only the AUG initiating codon in CHO cells is targeted to both the secretory pathway and the nucleus is in accordance with this possibility. [lxiv]

**Figure 5 Potential pathways used by PTHrP to gain access to the cytoplasm and nucleus.**

**a:** Secreted PTHrP undergoes endocytosis at the cell surface in a 'receptor'-mediated manner. Endocytosis could be mediated by PTH1R or a binding protein distinct from the receptor that recognises either the N-terminal domain or other regions of the PTHrP protein.
**b:** Initiation of translation of PTHrP mRNA downstream from the initiator methionine generates a signal peptide-truncated form of PTHrP. Such a protein localises within the cytoplasmic compartment because translocation through the membrane of the endoplasmic reticulum (ER) is now impaired.
**c:** PTHrP that has translocated into the ER lumen 'dislocates' back to the cytoplasm by the translocation unit or another transporter system (✹), and from there gains access to the nucleus.



### 1.7.b Mechanisms Regulating PTHrP Entry to the Nucleus

PTHrP can shuttle in both directions, between the nucleus and the cytoplasm via distinct import and export nuclear receptors.[lxxiii] This movement is dependent upon the intregrity of microtubules, consistent with a direct role for the cytoskeleton in protein transport to the nucleus.[lxxiv] Nuclear import is regulated by phosphorlyation of the PTHrP threonine residue at amino acid position 85 by cyclin-dependent kinase 2 p34,[lxiii] and is mediated almost exclusively by the saturable transport receptor importin β.[lxxv] A number of studies suggest that nuclear translocation of PTHrP is cell cycle dependent. Lam et al observed that PTHrP localises to the nucleus of cells in cultured HaCaT keratinocytes, at the $G_1$(quiescent) phase of the cell cycle, and was transported to the cytoplasm when cells divided.[lxxvi] In contrast Maasfelder et al reported that untransfected and PTHrP-overexpressing A10 vascular smooth muscle cells displayed immunoreactivity for PTHrP principally in the nuclei of cells that were in the process of division or completing cell division ($G_2$ or M).[lxxvii]

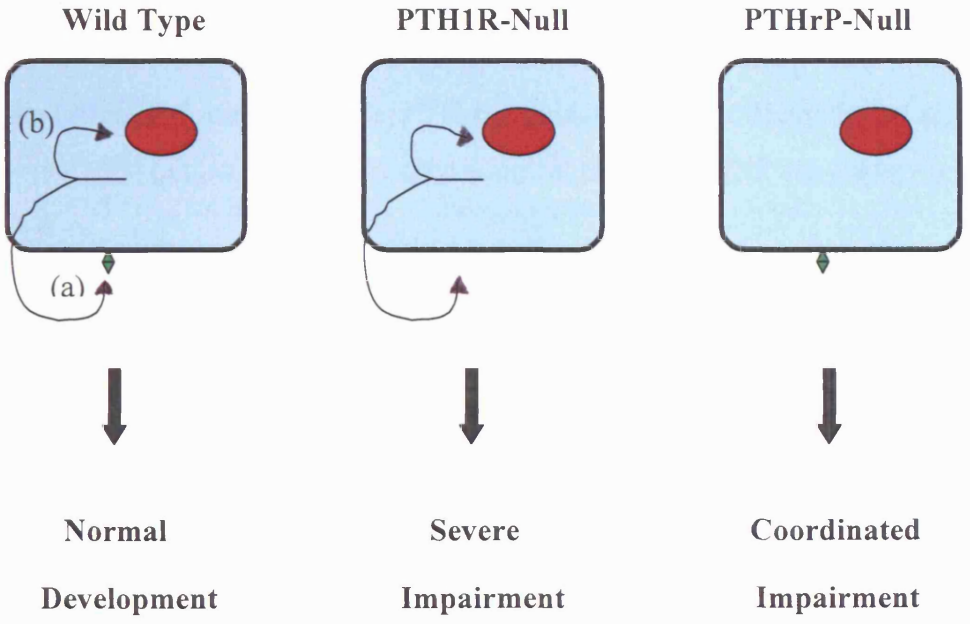
### 1.7.c Consequences of Nuclear Import of PTHrP

Endogenous PTHrP has been localized *in situ* to the dense fibrillar component of nucleoli, and the peptide has also been reported to bind homopolymeric and total cellular RNAs,[lxxviii] suggesting its potential involvement in regulating RNA metabolism. Several cellular effects have been associated with PTHrP nuclear import *in vitro*. It appears from available data that the physiological consequences of nuclear PTHrP entry vary from one cell type to another. Serum-deprived CFK2 chondrocytes and MCF-1 breast cancer cells over-expressing PTHrP with an intact NLS are protected from apoptosis.[lx, lxxix] In vascular smooth muscle cells (VSMC), the intracrine action of PTHrP is to activate cellular proliferation.[lxxvii] Interestingly, in these cells exogenous (i.e. paracrine/autocrine) PTHrP inhibits cell proliferation, indicating that the secreted and nuclear forms of PTHrP may have distinct, and at times, completely opposite effects on cellular function. PTHrP has also been demonstrated in prostate cells via an intracrine pathway, independent of the classical NLS, to produce production of IL-8.[lxxx] IL-8 is a potent angiogenic factor that contributes to tumourigenic activity in several cancers, and so PTHrP may mediate its effects on prostate cancer tumour progression via an intracrine pathway.

*In vivo* evidence from gene targeting studies supports the dual function of PTHrP. Mice that are homozygous for PTHrP gene ablation die in the early post natal period from lethal defects associated with premature terminal differentiation and calcification of chondrocytes.[lxxxi,lxxxii] In contrast, animals that are homozygous for PTH1R gene ablation exhibit a more severe phenotype characterised by early embryonic lethality (day 14.5 of gestation)[lxxxiii] in association with abrupt cardiomyocyte death.[lxxxiv] The less severe phenotype seen in the PTHrP gene ablated mouse is unlikely to be explained by circulating PTH acting via PTH1R to compensate for PTHrP deficiency, as PTH expression does not normally occur in the developing embryo until day 15.5, whereas PTHrP expression is widespread at that time. The more severe phenotype seen in the PTH1R deficient mice may reflect the dual function of PTHrP. It is likely that normal embryonic development requires the coordinated activity between the amino-terminal PTHrP acting on the cell surface PTH1R (autocrine/paracrine pathway) and the intracellular formating at the nucleus (intracrine pathway).[lxvii] In the absence of PTH1R, the unopposed nuclear events lead to severely dysregulated cell growth and/or differentiation, and therefore the early embryonic lethality of the receptor negative mutants. In contrast, in the absence of PTHrP, both receptor-mediated and nuclear activities are eliminated, leading to a more 'coordinated' cellular dysregulation, and hence less severe phenotype (Figure 6).

**Figure 6 Illustration of the co-ordinated activity of the autocrine/paracrine and intracrine pathways of PTHrP.**

In normal cells (wild type), the biological actions elicited by PTHrP require the coordinated activity between its **(a)** autocrine/paracrine pathway and **(b)** intracrine pathway.
In PTH1R deficient cells, extracellular signalling is absent, whereas the nuclear effects proceed unabated. This leads to severely dysregulated cell function and to the more severe phenotype of PTH1R-null animals.
In contrast, in ligand-deficient cells, both PTHrP signally pathways are abolished, leading to a more 'coordinated' functional disruption.



## 1.8 Physiological Effects of PTHrP

It is clear that PTHrP is produced in almost every tissue or organ in the body at some stage in foetal and adult development, and that PTHrP and PTH1R expression in early life is essential for normal development and survival. Insights into the variety of physiological roles of locally produced PTHrP as an autocrine/paracrine and intracrine factor have emerged from gene knock-out experiments and other approaches. Several common themes emerge from examining the physiological roles of PTHrP, namely: PTHrP may play more than one kind of role in a given tissue; PTHrP is a 'pro-hormone', and it is therefore possible for one secretory form of the peptide to have one effect whilst another secretory or intracrine form has another; in several tissues PTHrP has a role in transepithelial calcium transport; PTHrP appears to be a potent smooth muscle relaxant, the production of which can be induced by mechanical stretch of smooth muscle; PTHrP regulates growth, differentiation and development; and in many tissues the physiological roles of PTHrP are unknown.

*Table 1 Established and emerging physiological roles for PTHrP classified by organ systems.*

| Organ System | Functions of PTHrP |
|---|---|
| Cartilage | Regulates endochondral bone development. Promotes chondrocyte proliferation and delays chondrocyte maturation and apoptosis.<br><br>The bipartite NLS in the 88-106 region targets PTHrP to the nucleus where it regulates cell differentiation and apoptosis via an intracrine pathway<br><br>A second NLS in the 147-150 region targets PTHrP to the nucleus where it determines intracrine regulatory effects of PTHrP (1-173) in human chondrocytes on PPi metabolism and matrix synthesis |
| Bone | Stimulates bone resorption via its interaction with PTH1R.<br><br>PTHrP (107-139) (osteostatin) inhibits osteoclastic bone resorption via an as yet unidentified receptor.<br><br>Regulates Pi transport in osteogenic cells.<br><br>Ensures tooth eruption by resorption of alveolar bone, allowing passage of the newly developed tooth. |
| Skin and<br><br>Hair follicles | Interacts with PTH1R to delay terminal differentiation of epidermal keratinocytes, keratinisation and apoptosis.<br><br>Promotes epidermal proliferation.<br><br>Decreases the number of hair follicles via epithelial-mesenchymal interactions. |
| Placenta | Expressed by syncytiotrophoblast, intermediate trophoblasts, cytotrophoblasts, and amniotic epithelium.<br><br>Mid-region PTHrP interacts with an as yet unidentified receptor to maintain materno-foetal gradients of calcium and magnesium.<br><br>Modulates trophoblastic growth and differentiation by interacting with calcium-binding proteins and calcium-sensing receptors. |
| Mammary gland<br><br>and<br><br>Lactation | Necessary in epithelial-mesenchymal interactions during embryonic mammary development leading to nipple development and branching morphogenesis.<br><br>Induced by prolactin during lactation and released into the maternal circulating blood where it promotes calcium transport from blood to milk, increases mammary blood flow, and regulates maternal and neonate calcium-Pi metabolism. |
| Smooth muscle | PTHrP is rapidly induced by vasoconstrictors and mechanical stretching and interacts with PTH1R to induce strong myorelaxant effects in a number of smooth muscle containing organs. |
| Blood vessels | PTHrP and PTH1R are present in vascular smooth muscle cells (VSMC) and endothelial cells. |

| | |
|---|---|
| Blood vessels<br><br>continued | PTHrP decreases vascular tone and blood pressure and is believed to regulate regional and systemic haemodynamics.<br><br>PTHrP (1-36) binds to PTH1R to inhibit VSMC proliferation, whereas the bipartite NLS (88-106) targets PTHrP to the cell nucleus to stimulate cell proliferation and delay apoptosis. |
| Heart | Regulates foetal heart development.<br><br>Exerts positive chronotropic effects and induces inotropic effects resulting from coronary dilatation.<br><br>Released by ventricular and atrial myocytes. |
| Kidney | Possible role in kidney maturation and glomerular development. Expressed with PTH1R in renal tubules, glomeruli, intrarenal arterioles and arteries.<br><br>Mimics all PTH- effects on tubules by interacting with PTH1R.<br><br>Decreases renal vascular resistance, renal blood flow and glomerular filtration rate.<br><br>Exhibits a proliferative effect on tubular cells and mesangial cells.<br><br>Interacts directly with juxtaglomerular cells to stimulate renin release. |
| Lung | Mediates lung development via an epithelial-mesenchymal paracrine loop. It is an autocrine inhibitor of growth and differentiation of alveolar type II cells *in vivo* and *in vitro*. |
| Uterus | Upregulated by mechanical stretching and oestrogen. Potentiated by oestrogen, PTHrP relaxes uterus and vascular smooth muscle.<br><br>May regulate decidualisation of endometrial stromal cells.<br><br>May modulate implantation of the fertilised ovum and retention of the embryo. Inhibits oxytocin-stimulated activity during pregnancy and prevents preterm labour. |
| Gastrointestinal | Expressed with PTH1R in many structuro-functional regions of the gastrointestinal tract.<br><br>Relaxes the smooth muscle layers by interacting with vasoactive intestinal factor. Expression increased in smooth muscle layers in response to distension.<br><br>Induces intestinal calcium transport. |
| Liver | Possible foetal hepatic developmental factor regulating hepatogenesis that is extinguished postnatally.<br><br>Re-expressed during liver regeneration and endotoxic shock. |
| Pancreas | Produced with PTH1R in beta cells in which it mediates intracellular calcium.<br><br>Delays beta cell apoptotic death, increase beta cell growth and insulin secretion. |
| Parathyroid and | Produced and released by the oxyphil cell lineage in adult thyroid and parathyroid glands. |

| | |
|---|---|
| Thyroid glands | Enhances low-calcium stimulated PTH secretion *in vivo* and *in vitro*. |
| Ovary and Testis | PTHrP and PTH1R are expressed in the foetal and adult ovary and testis, but the function is unknown. |
| Prostate | PTHrP but not PTH1R is expressed by the neuroendocrine cells of prostatic epithelium and is present in seminal plasma. Its role is unknown. |
| Central nervous system | Expressed with PTH1R in a number of areas.<br>Possible involvement with astrocyte differentiation.<br>Protects neurons from glutamate-induced exitotoxicity.<br>PTHrP gene is expressed under conditions that promote the survival of neurons.<br>Displays a central pressor effect in conscious rats. |

Adapted from Clemens et al, 2001.[xxvi]

## 1.9 Expression and Effect of PTHrP in Tumour Systems

HHM is the most common paraneoplastic syndrome and occurs in up to twenty percent of patients with solid tumours. PTHrP is also expressed by tumours that are not associated with hypercalcaemia, for example prostatic adenocarcinoma, and recent evidence suggests that in addition to its endocrine effects, PTHrP may have an autocrine/paracrine role, and intracrine effects in these tumour systems, in which the peptide is thought to influence tumour growth. Table 2 summaries the tumour types in which PTHrP is known to be expressed, and in some cases has an established effect on tumour cell growth, apoptosis or differentiation.

*Table 2 Tumours in which PTHrP expression has been established, and the emerging effects of PTHrP in these tumours.*

| Tissue | Tumour Expression of PTHrP and Possible Role | References |
|---|---|---|
| Bone Metastases | When expressed by bone metastases, PTHrP enhances osteoblastic receptor activation of NF-κB ligand (RANKL), and diminishes osteoprotegrin (OPG) expression; RANKL and OPG are key regulators of osteoclast formation and inhibition respectively. | lxxxv, lxxxvi |
| Skin | Expressed by squamous cell carcinomas (SCC) and in melanomas. Humoral hypercalcaemia of malignancy (HHM) occurs in patients with advanced melanoma and rarely in cutaneous SCC. | lxxxvii, lxxxviii,lxxxix |
| Breast | HHM is common in patients with breast carcinoma. Well documented expression in the majority of breast adenocarcinoma, and increased expression in bone metastases, which may provide a selective growth advantage in bone. | xc, xci, xcii, xxiii |
| Kidney | Expressed in 95% of clear cell renal carcinomas, and via PTH1R is an essential growth and survival factor. | xciii, xciv, xcv |
| Lung | HHM is common in squamous cell lung carcinoma. Common expression in all types of lung cancer, particularly in more advanced, aggressive tumours. Exerts protective effects against apoptosis and hence may contribute to lung cancer cell survival and promote cancer progression. Serum PTHrP may predict survival. | xcvi, xcvii, xcviii |
| Gastrointestinal tract | Expressed by colon and gastric cancer. Elevated in serum of some patients with gastroesophageal carcinoma. | xcix, c, ci |
| Liver/Biliary tree | Expression by human cholangiocarcinoma. The first case of malignancy-associated hypercalcaemia was reported in a patient with cholangiocarcinoma. | cii, ciii |
| Exocrine pancreas | Expressed by pancreatic adenocarcinoma. | civ |
| Endocrine system | Expressed by pancreatic endocrine tumours (PET). There are several reported cases of HHM in patients with PET. Expressed in parathyroid adenomas, medullary thyroid tumours, phaeochromocytomas, adrenocortical carcinomas, and pituitary tumours. | cv, cvi, cvii |
| Bladder | Expressed by bladder carcinoma. | cviii |

| | | |
|---|---|---|
| Placenta | PTHrP is produced by chorioangioma, choriocarcinomas, and hydatidiform moles. | cix, cx |
| Uterus | Expressed by carcinoma of the uterine cervix. | cxi |
| Ovary | Expressed by ovarian carcinoma in which HHM is relatively common. | cxii, cxiii |
| Prostate | Commonly expressed by prostate carcinoma, in which it regulates tumour progression, especially in bone. HHM occurs rarely in these patients. | cxiv, cxv |
| Central Nervous System | Expressed by glial tumours, astrocytomas, and meningiomas. In glial tumours high expression is associated with reduced local control and survival after tumour irradiation. | cxvi, cxvii, cxviii |
| Immune System | Expressed in lymphoma, leukaemia, and myeloma, in which HHM is common. | cxix, cxx |

## 1.10 Rationale of Thesis

Expression of PTHrP protein by tumour cells has previously been described by Bouvet et al in resection specimens from normocalcaemic patients with pancreatic adenocarcinoma.[civ] This group also detected PTHrP protein in western immunoblots of lysates from pancreatic adenocarcinoma cell lines. The expression of PTH1R by human pancreatic adenocarcinoma however has not previously been confirmed.

The purpose of the first part of this study was to examine by immunohistochemistry the expression of amino-terminal PTHrP and PTH1R, using a novel monoclonal antibody against the amino-terminal PTHrP [anti-PTHrP (1-10)] and an established anti-PTH1R antibody, in resection specimens from patients with well defined adenocarcinoma of the pancreas who were normocalcaemic pre-operatively. The disadvantage of using immunohistochemistry is that precise localization to a particular cell can be difficult and it is uncertain whether the cells detected by this method are the origin or target of the protein PTHrP, or rather have non-specifically absorbed the secreted protein. This question can be clarified by using in situ hybridization or real time polymerase chain reaction (RT-PCR) to detect PTHrP and PTH1R mRNA. However current available techniques are insensitive on paraffin-embedded archival tissue, poorly localizing, and time consuming. Acquisition, with informed patient consent, of new resection specimens of pancreatic adenocarcinoma for preservation in cryosection in sufficient numbers for this study, would have taken many years. This is because patients with adenocarcinoma of the pancreas usually present at an advanced stage, and therefore only a small proportion of patients go on to potentially curative surgery. Additionally new radiological techniques such as endoscopic ultrasonography and high resolution computer tomography have improved tumour staging to the extent that even fewer patients go to surgery. PTHrP and PTH1R mRNA was therefore not assessed in these tissues. Confirmation that PTHrP and PTH1R protein is expressed by tumour cells was assessed by performing immunohistochemistry on cytospin blocks of cultured cells, and western immunoblotting of whole cell lysates, from pancreatic adenocarcinoma cell lines.

Neuroendocrine tumours express a number of peptides, and peptide receptors, with associated humoral mechanisms. PTHrP is produced by beta-cells in the pancreatic

islets, inducing cytosolic calcium responses, and regulating cell growth. In the second part of this study, the immunohistochemical expression of amino-terminal PTHrP and PTH1R was assessed in paraffin-embedded tumour resection specimens from patients with a variety of neuroendocrine tumours who did not have hypercalaemia. PTHrP and PTH1R expression in neuroendocrine tumour cell lines was investigated by immunohistochemistry and western immunoblotting. Further, any potential relationship between the expression of PTHrP/PTH1R and cellular proliferation was evaluated by measuring the immunohistochemical expression of nuclear Ki67 by tumour cells in the resection specimens, and correlating this with PTHrP/PTH1R expression.

Preliminary work suggested that PTHrP is expressed in human hepatocellular carcinoma.[cxxi] Using the above monoclonal anti-PTHrP (1-10) antibody, PTHrP was detected by immunohistochemistry in hepatocellular carcinoma tumour cells in paraffin-embedded resection specimens. The third part of this study examines the immunohistochemical expression of amino-terminal PTHrP and PTH1R in a series of cases of hepatocellular carcinoma resection specimens from patients who were normocalcaemic. PTHrP and PTH1R protein expression was also assessed by immunohistochemistry on cultured cells, and western immunoblotting of cell lysates, from hepatocellular carcinoma cell lines.

The observation that PTHrP is expressed in foetal liver, expressed at a low level or not at all in adult liver, and then 'switched on' in hepatocellular carcinoma suggests that in liver, as in a number of other tissues, PTHrP may play a role as a regulatory factor for hepatocyte growth and differentiation. As the majority of patients who develop hepatocellular carcinoma develop it on a background of cirrhosis of varying aetiology, this suggests that cirrhosis itself is a pre-neoplastic condition. If amino-terminal PTHrP and PTH1R play a paracrine/autocrine role in the progression and differentiation of hepatocellular carcinoma, then amino-terminal PTHrP and PTH1R would be expected to be expressed by hepatocytes in cirrhotic liver, and in premalignant liver nodules. Therefore the purpose of the fourth part of the study was to investigate by immunohistochemistry the expression of amino-terminal PTHrP and PTH1R in resection specimens from human normal liver, cirrhotic liver (regenerating nodules), macroregenerative nodules within cirrhotic liver, dysplastic nodules, which

are putative hepatocellular carcinoma precursor lesions, and hepatocellular carcinoma. If the effect of amino-terminal PTHrP/PTH1R is to influence cell proliferation, then a correlation between it's expression and the cell proliferation marker Ki67 would be expected. Proliferative activity in the tissues was therefore evaluated by measuring the immunohistochemical expression of nuclear Ki-67 antigen by hepatocytes or tumour cells in the same resection specimens. Any relationship between amino-terminal PTHrP/PTH1R expression and cellular proliferation was assessed.

Finally, functionality and cellular localisation of PTH1R expressed by the hepatocellullar carcinoma cell line HepG2 and the positive control cell line, prostatic adenocarcinoma PC3, was assessed by examining the uptake of fluorescently labelled amino-terminal PTHrP (1-34) by these cells.

The expression of both PTHrP and PTH1R by the tumour types studied may imply a possible autocrine/paracrine role for PTHrP in these tumours. A correlation between PTHrP/PTH1R expression and cell proliferation may also imply a regulatory role for PTHrP/PTH1R in tumour growth.

# Chapter 2 EXPRESSION OF PARATHYROID HORMONE-RELATED PEPTIDE AND THE PARATHYROID HORMONE TYPE 1 RECEPTOR IN PANCREATIC ADENOCARCINOMA.

## 2.1 Introduction

Parathyroid hormone-related peptide (PTHrP) is normally produced in the pancreatic beta cell, and can induce intracellular responses in cultured beta cells, indicating that these cells may have a paracrine/autocrine role involving PTHrP.[liv] In vivo studies in mice, in which PTHrP was targeted to and overexpressed in the pancreatic beta cell, have shown evidence of growth-regulating properties of PTHrP.[cxxii, cxxiii] In these animals there was a progressive increase in pancreatic cell mass, islet number, and beta cell number.

There are a growing number of reports of PTHrP-secreting pancreatic endocrine tumours causing hypercalcaemia.[cxxiv, cxxv, cxxvi, cxxvii, cxxviii] Hypercalcaemia associated with exocrine pancreatic tumour has also been reported, and in a few case reports PTHrP has been localized by immunohistochemistry in the tumour.[cxxix, cxxx, cxxxi] Bouvet et al [civ] have shown that PTHrP is produced by human pancreatic cancer cell lines, and can be detected by immunohistochemistry in tumour cells of human pancreatic adenocarcinoma tumour specimens in normocalcaemic patients. This group did not investigate the expression of the PTH/PTHrP type 1 receptor (PTH1R) by the tumours or tumour cell lines.

The aims of these experiments were: 1) to investigate by immunohistochemistry the expression of PTHrP and its corresponding PTH1R in pancreatic adenocarcinoma tumour resection specimens from patients who were normocalcaemic at the time of resection; and 2) to investigate by immunohistochemistry and western immunoblotting the expression of PTHrP and PTH1R in pancreatic cancer cell lines.

## 2.2 Materials and Methods

### 2.2.a Optimisation of Antibodies

*2.2.a.1 Anti-PTHrP*

A novel mouse monoclonal anti-amino-terminal PTHrP antibody [PTHrP (1-10)] (Aphton Corporation, FL, USA) was optimised on paraffin-embedded tissues from regenerating liver and breast adenocarcinoma, which are well documented to express PTHrP [cxxxii, cxxxiii] (Appendices I and II). Dilutional studies with this antibody showed that the optimal dilution for immunohistochemistry was 1:1000.

*2.2.a.2 Anti-PTH1R*

Mouse monoclonal PTH1R antibody (Lab Vision Corporation, CA, USA), raised against amino acids 146-169 of the human PTH/PTHrP receptor, was optimised on paraffin-embedded tissue from the above cases of regenerating liver and breast adenocarcinoma (Appendix II). Breast adenocarcinoma is also well documented to express PTH1R.[cxxxiv] A dilution of 1:50 was the optimal concentration for these experiments.

### 2.2.b Patient Tissue Specimens and Cell Lines

*2.2.b.1 Patient Tissue Specimens*

Seventeen consecutive cases of well-characterised ductal pancreatic adenocarcinoma obtained at pancreatoduodenectomy between 1994 and 2003 were selected from the pathology archives of the Royal Free Hospital (Table 3). Tissue had been immediately formalin-fixed, routinely processed, and embedded in paraffin.

All of the patients had been normocalcaemic prior to their procedures. All patients had given consent for the use of the tissue for research, under ethical approval by the Royal Free Hospital ethics committee. The following clinicopathological details about

the tumour case were collected: tumour differentiation, histological evidence of local, perineural and vascular invasion, and the presence of lymph node metastases.
Sequential sections (5μm thick) were cut with a microtome from the formalin-fixed, paraffin-embedded tissues, mounted on APES-coated slides, and dried overnight at 60°C.

*2.2.b.2 Cell Lines*

The following pancreatic carcinoma cells lines were studied: Panc-1, BxPC3, and AR42J. Panc-1 and BxPC3 are both human pancreatic carcinoma cell lines of ductular origin (Panc-1 and BxPC3 are poorly differentiated and moderately differentiated tumour cell lines respectively).[cxxxv] AR42J cells are derived from a rat pancreatic adenocarcinoma.[cxxxvi] All 3 cell lines were obtained from the European Collection of Animal Cell Cultures (ECACC, Porton Down, UK). Panc-1 cells were cultured in Dulbecco's Modified Eagles Medium (DMEM) (Sigma Chemical, Poole, UK) containing 10% foetal calf serum (FCS) (Sigma) and 2mM L-glutamine (Sigma). BxPC3 and AR42J were cultured in RPMI-1640 medium (Sigma Chemical, Poole, UK) containing 10% FCS (Sigma) and 2mM glutamine (Sigma). All cells were grown at 37°C in a humidified 95% air - 5% $CO_2$ atmosphere, and medium was changed twice weekly.

The human prostate cancer cell line PC3, which is known to express and secrete PTHrP[cxxxvii] and express a functional PTH1R,[cxxxviii] was chosen as a positive control cell line. PC3 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), and cultured in RPMI-1640 containing 10% FCS and 2mM glutamine.

Cytospin blocks were prepared for each cell line from cells that had been grown to 80% confluence. Sequential sections (5μm thick) were cut with a microtome from the formalin-fixed, paraffin-embedded tissues, mounted on APES-coated slides, and dried overnight at 60°C.

### 2.2.c Immunohistochemistry

Tissue sections and cell cytospin sections were dewaxed in xylene (Chemicon Europe, Limited, Hampshire, UK) for 10 minutes, rehydrated in 100% alcohol and then rinsed in double-distilled water for 5 minutes. Immunohistochemical localization of PTHrP and PTH1R was performed by the alkaline phosphatase anti-alkaline phosphatase (APAAP) method (Appendix III). All experiments were performed at room temperature, and were validated by the inclusion in each run of a positive control (breast adenocarcinoma) section for each antibody and parallel negative control sections for each tissue tested. Negative control sections were made by substitution of the primary antibody with normal mouse serum. For the anti-PTHrP antibody, specificity of staining was further validated by the inclusion of a parallel section of tissue that was incubated with a primary antibody solution of the same concentration of antibody (1 in 1000 dilution) that had been pre-absorbed overnight with excess immunizing peptide (0.25mg PTHrP [1-10]).

### 2.2.d Evaluation of Immunohistochemistry Studies

Sections were examined by light microscopy and scored by two independent observers. Cells were considered positively stained if cytoplasm, cell membrane, or nucleus was distinctly coloured compared with background, and if staining was absent from adjacent negative control sections.

For tumour sections and cell lines the intensity of staining was compared to that of the positive control for each antibody in each run. The intensity of staining was graded on a scale from 0 to 3 where, 0 indicated no staining; 1 indicated weak intensity of staining; 2, moderately intense staining; and 3, most intense staining. Sections were also given a score to grade the extent of staining, such that less that 25% positive cells = 1; 25-75% cells = 2; and more than 75% = 3. To obtain a numerical score, the intensity of staining was multiplied by the number of cells stained. This scoring system has been previously validated.[cxxxiii, cxxxix] Scores were used to separate the

positive results into weak (1 and 2), moderate (3 and 4), and strong (6 and 9) categories.

### 2.2.e Statistical Methods

Statistical analyses were performed using the Spearman rank correlation test, and the Mann-Whitney U non-parametric test for group differences. These were carried out using SPSS, version 11.0 (2001) (SPSS Inc., Chicago, USA). A *P* value of less than 0.05 was considered to be statistically significant.

### 2.2.f Western Immunoblotting

*2.2.f.1 Preparation of cell lysates*

Cells from the following cell lines were cultured in T-75 flasks: AR42J, BxPC3, Panc-1 and PC3. After washing with phosphate buffered saline (PBS) the cells were lysed in 800mM Tris buffer pH 6.8 containing 5% SDS and 100mM phenylmethyl-sulfonyl fluoride. Protein content in the lysates was measured using a modified Lowry assay (Bio-Rad DC Protein Assay, Bio-Rad, CA, USA).

*2.2.f.2 Immunoblotting*

Prepared lysates were boiled for 4 minutes with bromophenol blue and 30% glycerol. A volume of lysate corresponding to 10 μg of protein was loaded onto Laemmli SDS-polyacrylamide gels. Western blotting conditions and gels were optimized in order to detect bands below 6kD [PTHrP (1-34): $M_r$ = 4017.61]. The gels were run at 100V in Tris-glycine buffer containing 0.1% SDS until the dye front reached the bottom of the gel. A low molecular weight range protein standard (Sigma Marker™, Sigma) was also loaded onto each gel. After separation, the proteins were electrotransferred in cooled transfer buffer to nitrocellulose at 250mA (100-120V) over 60 minutes. The membranes were blocked in PBS containing 5% bovine serum albumin (BSA) (Sigma) and 0.1% Tween 20 (Lab Vision Corporation, CA, USA) for 30 minutes at room temperature, and then incubated with primary antibody overnight at 4°C. After three 5- minute washes in PBS/0.1% Tween 20, the membranes were incubated with goat anti-rabbit/mouse biotin (DAKO, Copenhagen, Denmark) at a 1:200

dilution in the same buffer for 1 hour. The membranes were washed again three times in PBS/0.1% Tween 20 and then incubated with streptavidin/biotin-peroxidase complex (1:200 dilution, DAKO) for 30 minutes. Peroxidase activity was revealed using a diamino-benzidine/hydrogen peroxide solution (Vector VIP, Vector Laboratories, UK).

*2.2.f.3 Primary Antibodies*

The following primary antibodies were used:

PTHrP (1-34) antibody

Rabbit polyclonal antibody [Bachem (UK) Ltd., Merseyside, UK], raised against the amino-terminal sequence of PTHrP, was used at a dilution of 1 in 200 in PBS. As an additional control, 15μg of immunizing PTHrP peptide [Bachem (UK) Ltd] was loaded and included in each run. The results were validated by the inclusion of parallel blots which had been incubated with a primary antibody solution of the same concentration of antibody that had been pre-absorbed overnight with excess immunizing peptide [0.1mg PTHrP (1-34)]. Bands were considered to represent PTHrP peptides only if they were abolished or significantly attenuated by pre-absorption.

PTHrP (38-64) antibody

Mouse monoclonal antibody (Oncogene Research Products, CA, USA), raised against an amino acid sequence within the mid-fragment PTHrP, was used at a concentration of 5μg/ml. The immunizing peptide was not commercially available.

PTH1R antibody

The following amendments were made prior to immunoblotting with this antibody which was used at a dilution of 1 in 10: cell lysates were incubated with bromophenol blue and 30% glycerol at room temperature; 10% SDS-polyacrylamide gels were used with Full Range Rainbow™ (Amersham Biosciences UK Limited, Buckinghamshire, UK) recombinant protein molecular weight markers.

## 2.3 Results

### 2.3.a Immunohistochemistry on Patient Tissue Sections and Cell Lines

*2.3.a.1 Patient Tissue Sections*

PTHrP

Of the 17 cases of pancreatic adenocarcinoma tested, 16 showed positive tumour cell staining for PTHrP [median score 6 (range 0-6)] (Table 3). Staining of tumour cells was predominantly cytoplasmic, and occurred in a background of stromal fibrosis typical of pancreatic adenocarcinoma (Figure 7). In some of the tumour resection specimens, nuclear staining in tumour cells was also seen (case numbers 6, 10, and 17). As expected, there was positive staining for PTHrP in adjacent normal pancreatic islet cells, and some normal exocrine ducts and ductules.[cvi] Negative controls (no primary antibody and competition with excess PTHrP) revealed no positive staining.

PTH1R

In 15 of the 17 pancreatic adenocarcinoma specimens, tumour cells showed positive cytoplasmic staining for PTH1R [median score 3 (range 0-6)] (Table 3; Figure 8). There was variable cytoplasmic staining for PTH1R in adjacent normal pancreatic islet cells and ducts. Nuclear staining was also observed in some of the tumour resection specimens (case numbers 6, 10, and 17). All staining for PTH1R was abolished by substitution of normal mouse serum for primary antibody.

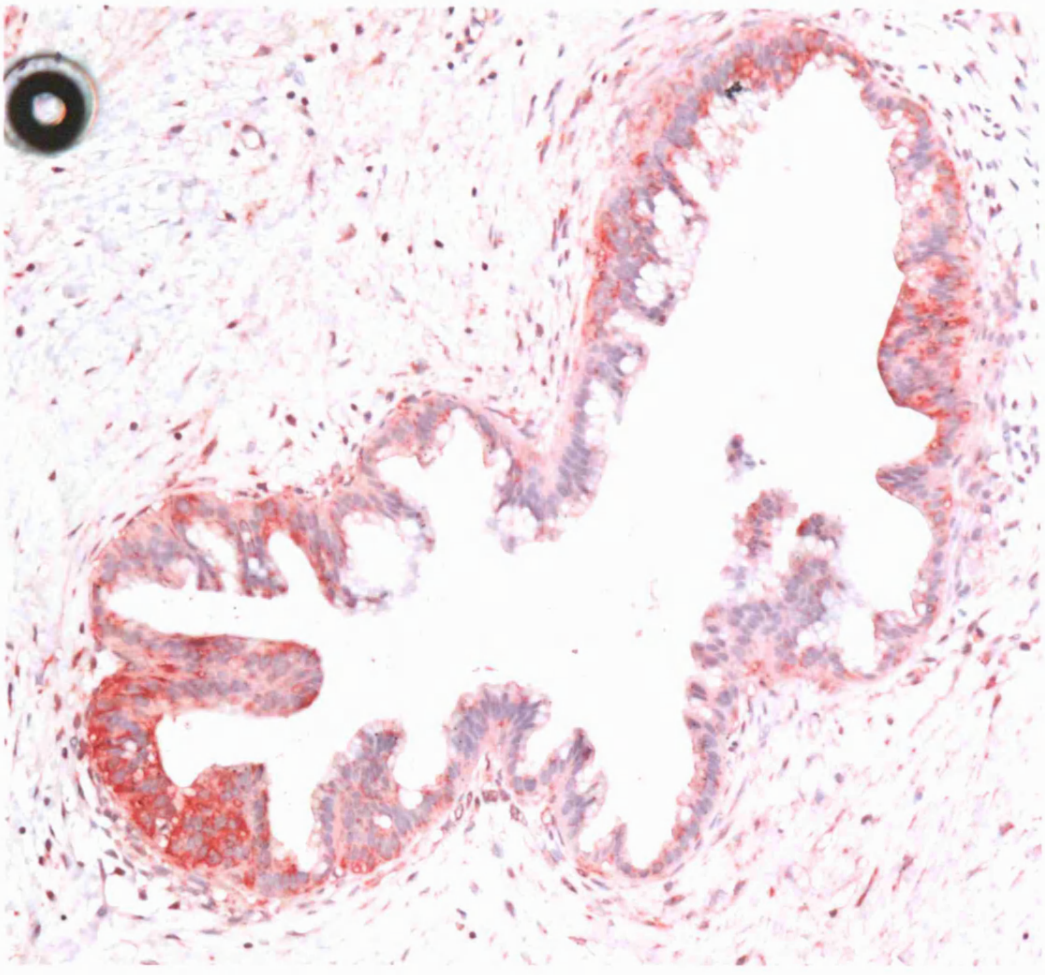


Figure 7 (a)

Figure 7 Immunohistochemistry showing positive tumour cell staining for PTHrP in resection specimens of human pancreatic adenocarcinoma.

The images are from four different cases of pancreatic adenocarcinoma, and are representative of the results obtained. Positive staining appears as a red precipitate. Figures (a), (b), and (c) demonstrate positive cytoplasmic staining (photographed at magnification x100). Figure (d) shows positive nuclear and patchy cytoplasmic staining (photographed at magnification x200).

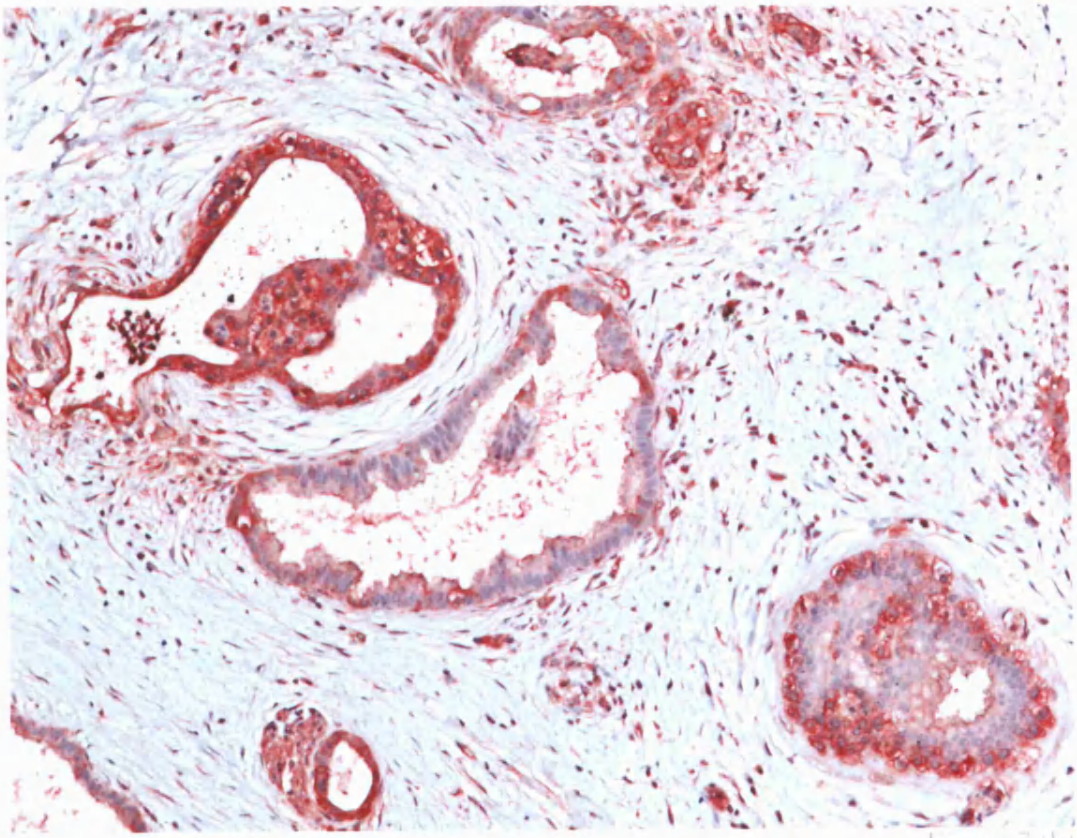


Figure 7 (b)

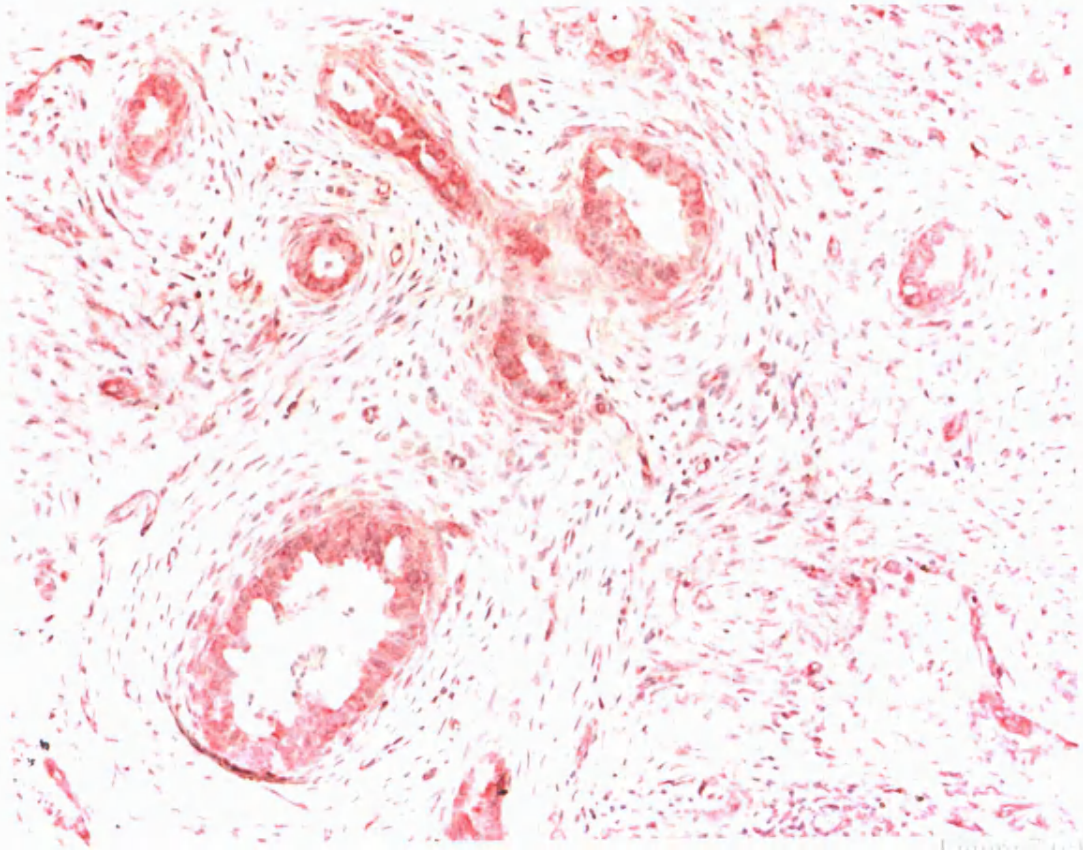


Figure 7 (c)

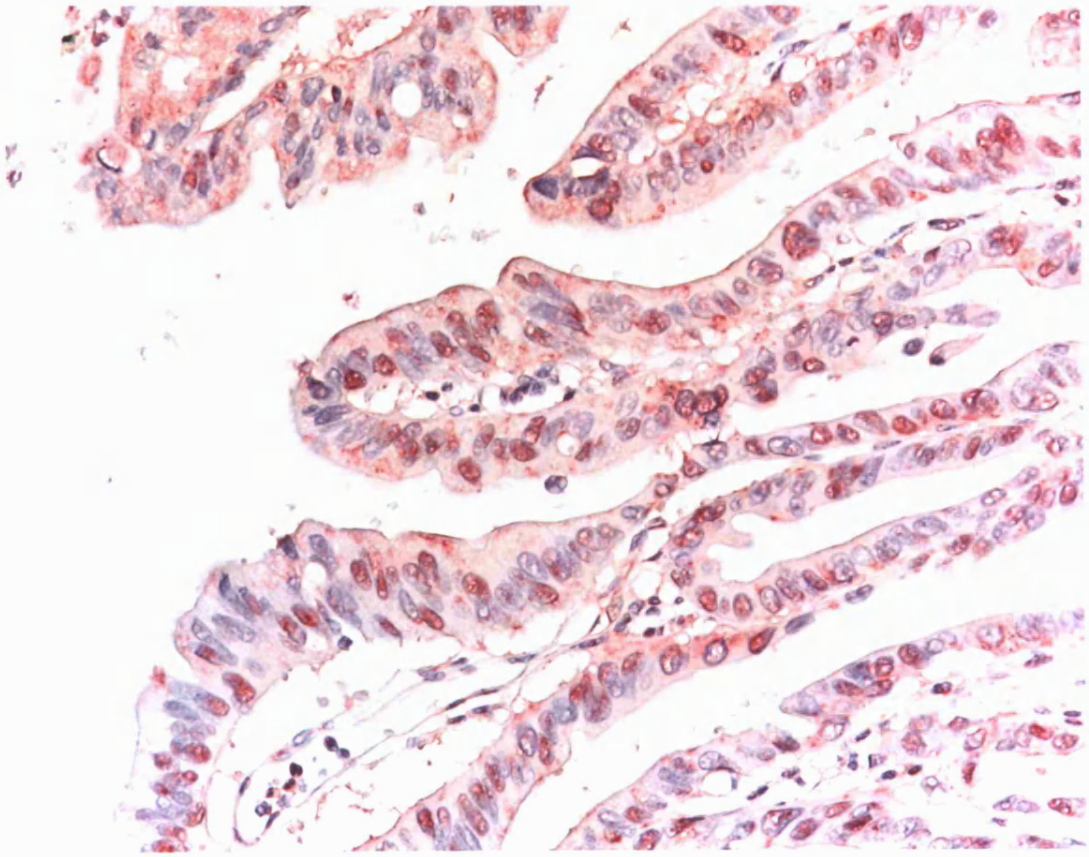


Figure 7 (d)

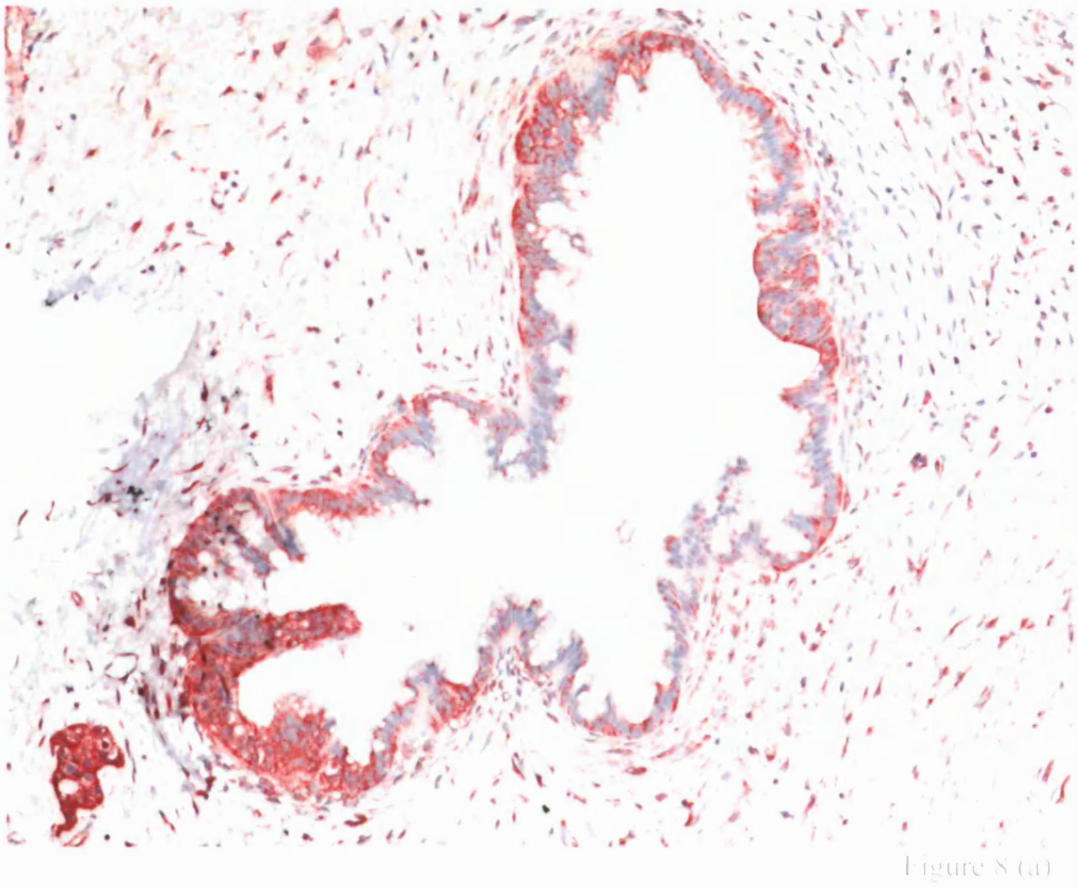



Figure 8 Immunohistochemistry showing positive tumour cell staining for PTH1R in resection specimens of human pancreatic adenocarcinoma.

The images are from the same four cases of pancreatic adenocarcinoma as shown in Figure 7, and are representative of the results obtained. Positive staining appears as a red precipitate. Figure (a) shows positive cytoplasmic and membranous staining (Photographed at magnification x100). Figures (b) and (c) show positive cytoplasmic staining (photographed at magnification x100). Figure (d) shows positive nuclear and cytoplasmic staining (photographed at magnification x200).

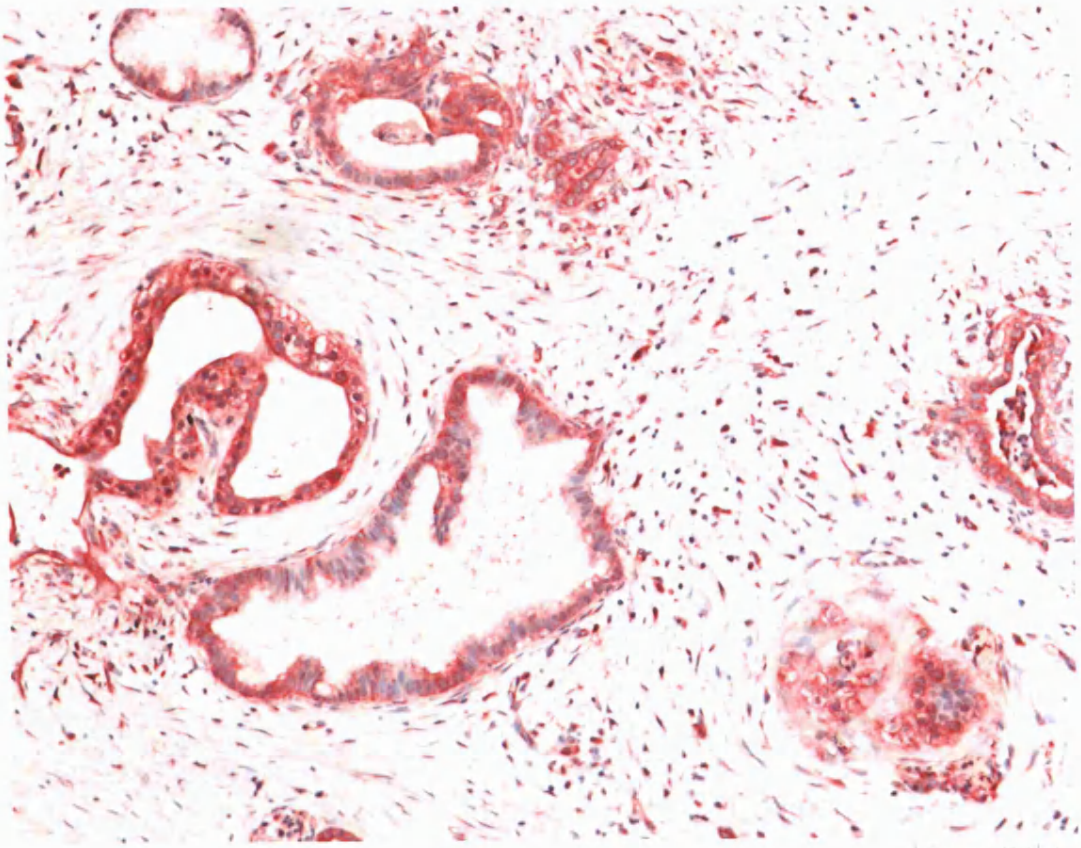


Figure 8 (b)

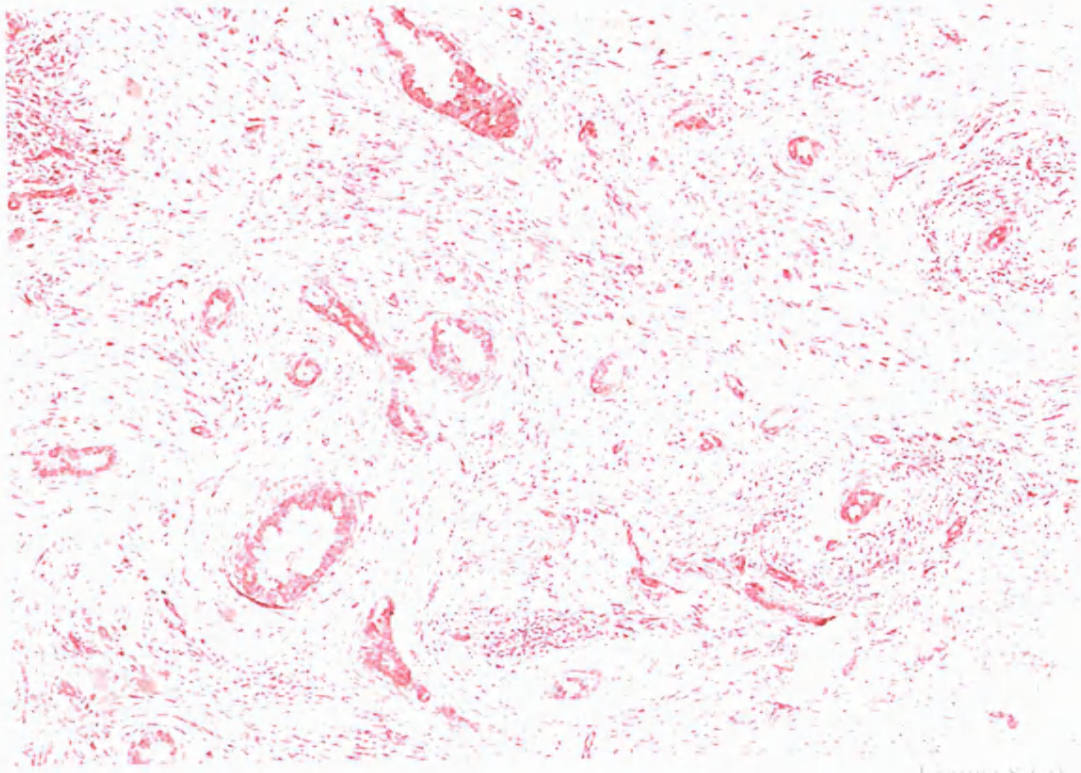


Figure 8 (c)

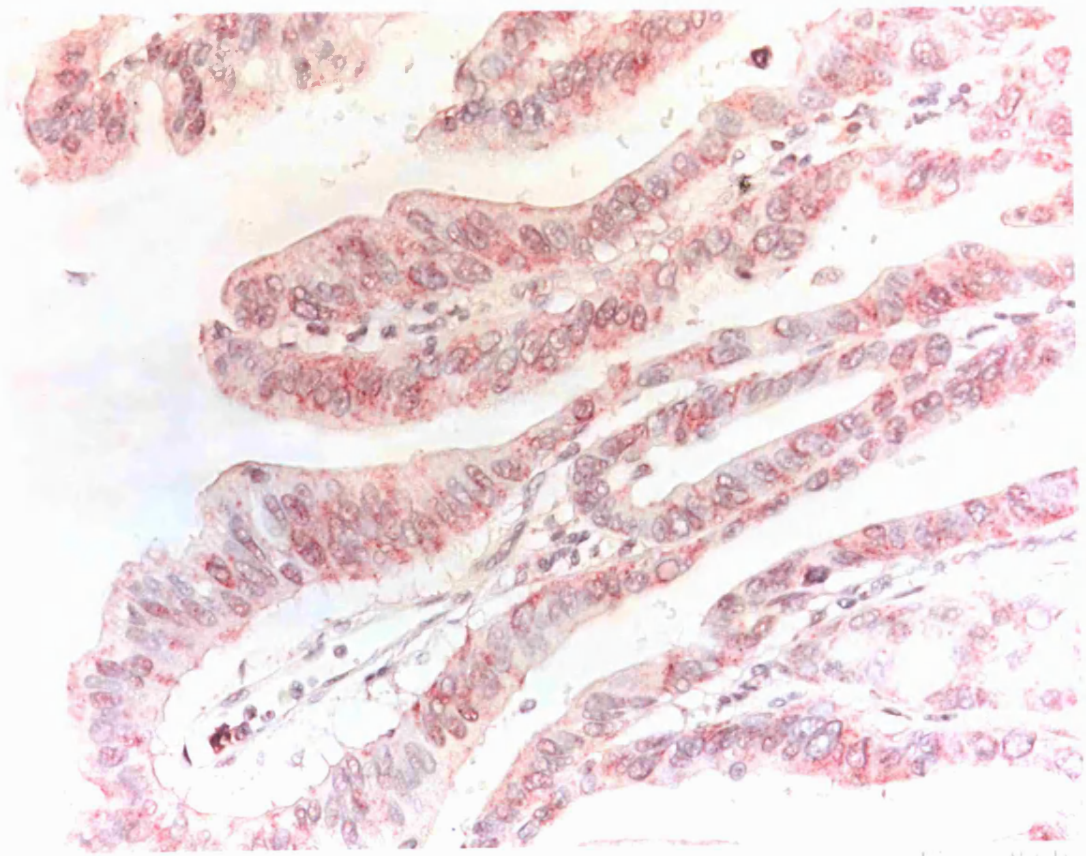


Figure 8 (d)

*Table 3 Histological details and immunohistological expression of PTHrP and PTH1R in specimens from 17 patients with pancreatic adenocarcinoma.*

| Case No. | Age (years) | Sex | Differentiation of Tumour | Tumour invasion | | | | Tumour cell staining | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Local Tissue | Vascular | Perineural | Lymph node | PTHrP (0-9) | PTH1R (0-9) |
| 1 | 56 | M | in situ duct carcinoma | no | no | no | no | 6 | 6 |
| 2 | 61 | F | moderate | yes | yes | yes | no | 6 | 4 |
| 3 | 68 | M | moderate | yes | yes | yes | no | 3 | 4 |
| 4 | 62 | M | moderate | yes | no | yes | yes | 1 | 0 |
| 5 | 74 | F | well | yes | no | yes | no | 4 | 2 |
| 6 | 65 | M | moderate | yes | yes | yes | yes | 4 | 6 |
| 7 | 64 | M | variable (moderate/well) | multifocal | yes | yes | no | 2 | 0 |
| 8 | 48 | F | well | yes | yes | yes | yes | 1 | 1 |
| 9 | 81 | M | well | yes | no | no | yes | 6 | 3 |
| 10 | 74 | F | moderate | yes | yes | yes | yes | 6 | 4 |
| 11 | 65 | F | well | yes | no | yes | no | 6 | 4 |
| 12 | 64 | M | well | yes | no | yes | yes | 6 | 2 |
| 13 | 47 | F | moderate | yes | no | yes | yes | 6 | 3 |
| 14 | 54 | F | moderate | yes | no | yes | no | 6 | 4 |
| 15 | 69 | F | moderate | yes | yes | yes | no | 6 | 4 |
| 16 | 45 | M | moderate | yes | yes | yes | yes | 0 | 3 |
| 17 | 55 | M | moderate | yes | yes | yes | no | 4 | 3 |

**Legend:** M, male; F, female

In 14 of the 17 cases of pancreatic carcinoma, there was positive staining for both PTHrP and PTH1R. There was a positive correlation between the tumour staining scores for PTHrP and for PTH1R ($R = 0.527$, $p = 0.025$). There was no significant difference between the staining scores for either PTHrP or PTH1R in the well

differentiated tumours compared to the moderately differentiated tumours (there were no poorly differentiated tumours in this series). There was no significant difference in tumour staining for either PTHrP or PTH1R in tumours with or without histological evidence of local, vascular or perineural invasion.

*2.3.a.2 Cell Lines*

Preliminary studies on PC3 cell cytospin sections showed strong cytoplasmic staining for PTHrP in all cells (score 9). Nuclear staining was also seen in 5-10% of cells. Positive staining of moderate intensity was observed for PTH1R in the cytoplasm and membrane of all cells (score 6).

PTHrP

PTHrP was localised by immunohistochemical staining in the cytoplasm of all 3 cell lines tested (Table 4, Figure 11). Nuclear staining was observed in 1-5% of BxPC3 cells (Figure 9). Preabsorption of the antibody with the immunizing peptide abolished all staining.

PTH1R

PTH1R was localised to the membrane and cytoplasm in BxPC3 and Panc-1 cells (Table 4). Nuclear staining for PTH1R was also seen in 1% of BxPC3 cells (Figure 10). There was no staining for PTH1R in AR42J cells (Figure 11). All staining for PTH1R was abolished by substitution of normal mouse serum for primary antibody.

Figure 9 Immunohistochemistry showing nuclear and cytoplasmic staining for PTHrP in the human pancreatic carcinoma cell line BxPC3 (original magnification x 100)

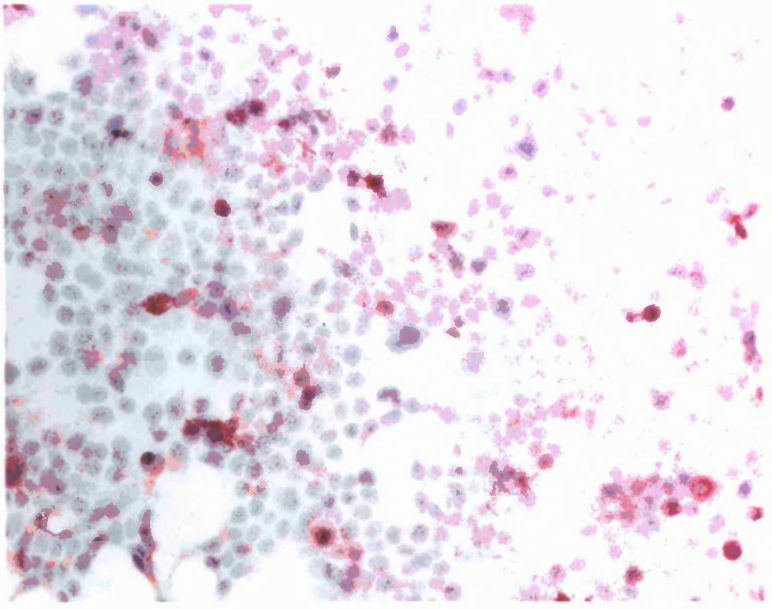


Figure 10 Immunohistochemistry showing cytoplasmic and nuclear staining for PTH1R in the human pancreatic carcinoma cell line BxPC3 (original magnification x 100)

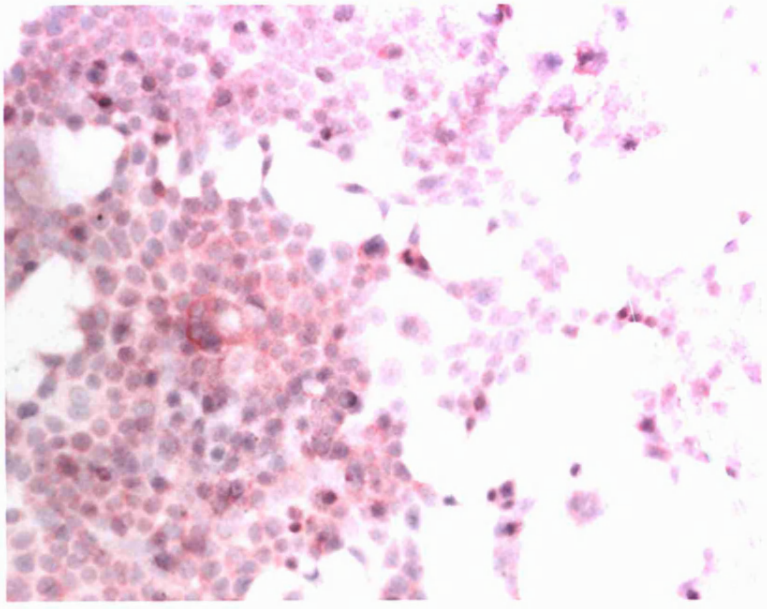


Figure 11 Immunohistochemistry showing cytoplasmic staining for PTHrP (figure a) and no staining for PTH1R (figure b) in cell cytospins of the rat pancreatic cell line AR42J. (Original magnification x 200)

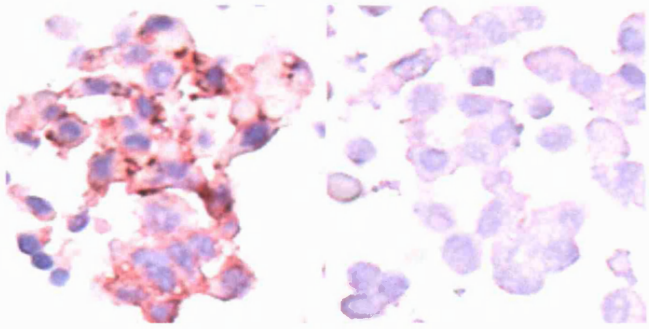


Table 4 Immunohistochemical expression of PTHrP and PTH1R in the pancreatic cell lines studied.

| **Cell line** | **Intensity and location of immunostaining for PTHrP** | **Intensity and location of immunostaining for PTH1R** |
|---|---|---|
| AR42J | 9 (C) | 0 |
| BxPC3 | 6 (C & N) | 6 (N, C, M) |
| Panc-1 | 6 (C) | 4 (C & M) |

**Legend:** C = cytoplasmic, M = membranous, N = nuclear

The immunoreactive product was scored between 0 and 9. To obtain a numerical score, the intensity of staining (scored from 0 to 3) was multiplied by the percentage of cells stained (scored from 0 to 3).

### 2.3.b Western Immunoblotting

PTHrP (1-34)

A distinct band that was abolished by pre-absorption was present for the control PTHrP (1-34) peptide at approximately 5 kDa. Distinct bands that were abolished by pre-absorption were present in the PC3, AR42J and Panc-1 cell lysates at 17 kDa, 35 kDa, and 45 kDa, and in the BxPC3 cell lysate at 35 kDa and 45 kDa (Figure 12a).

PTHrP (38-64)

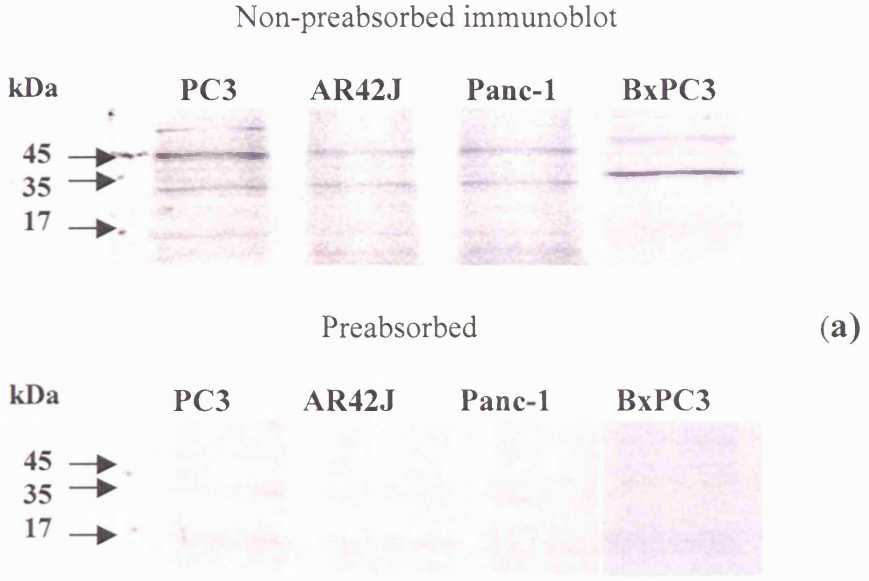
A distinct two-band pattern with proteins at 15- and 50 kDa was noted in the PC3 and AR42J lysates, and at 35 kDa and 50 kDa in the Panc-1 and BxPC3 lysates.

PTH1R

Bands were seen at 60 kDa in the PC3, Panc-1, and BxPC3 cell lines. Bands at 45 kDa were seen in AR42J, Panc-1, and PC3. Additional bands at 80- and 160 kDa were seen in the PC3 lysate.

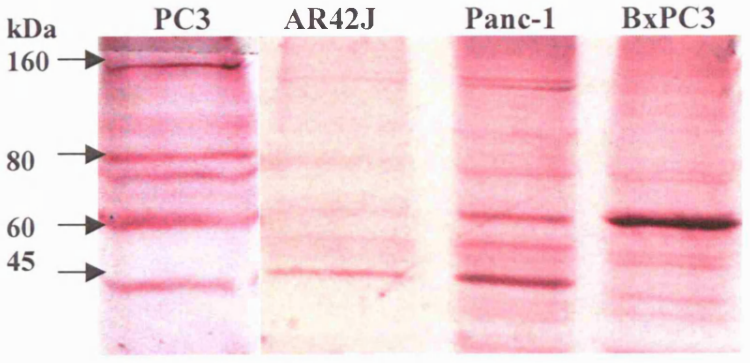
Figure 12 Western immunoblots for PTHrP (1-34) and PTH1R on whole cell lysates from pancreatic adenocarcinoma cell lines and prostate adenocarcinoma cell line PC3.

Figure (a) is a representative immunoblot for PTHrP (1-34). Bands that are attenuated by pre-absorption are seen at 17 kDa, 35 kDa, and 45 kDa in the PC3, AR42J and Panc-1 cell lysates, and in the BxPC3 cell lysate at 35 kDa and 45 kDa.




(a)

Figure (b) is a representative immunoblot for PTH1R. Distinct bands are seen at 60 kDa in the PC3, Panc-1, and BxPC3 cell lines. A band at 45 kDa only is seen in the rat adenocarcinoma cell line AR42J. This 45 kDa band is also present in the PC3 and Panc-1 lysate.




(b)

## 2.4 Conclusions

These results show that there is protein expression of PTHrP in all 3 pancreatic cancer cell lines and in the majority of the human pancreatic adenocarcinoma specimens that were studied. PTH1R protein is also highly expressed in human pancreatic adenocarcinoma tumour cells, but was not detected by immunohistochemistry or western immunoblotting in the rat adenocarcinoma cell line AR42J.

The 15-17 kDa protein bands detected by immunohistochemistry on the Western blots are appropriate sizes for PTHrP (1-139) and PTHrP (1-141) molecules (Mw 16-20 kDa).[cxl] The anti-PTHrP antibodies also detected peptides of approximately 35 kDa and 45-50 kDa, which may represent polymerisation of PTHrP molecules, or possibly represent glycosylated mature PTHrP, providing indirect evidence for PTHrP processing. Bouvet et al[civ] reported that they obtained 17 kDa, 34 kDa, and 44 kDa bands of immunoreactive PTHrP in BxPC3 and Panc-1 cell lysates, among other pancreatic cancer cell lines tested, using a monoclonal antibody to PTHrP (109-141). Other groups have also reported obtaining bands of similar sizes on western blots of proteins extracted from different tissues using rabbit polyclonal anti-PTHrP (1-34) and PTHrP (56-86).[cxl, cxli] Schlüter et al, using the same monoclonal PTHrP (38-64) antibody used in this study to immunoprecipitate PTHrP from rat coronary endothelial cells, also detected a single protein band with an apparent molecular weight of about 50 kDa.[cxlii] Utilising a digoxigenin-glycan detection assay, indicating glycosylation of proteins, this group confirmed that the higher molecular weight PTHrP was modified by glycosylation, and postulated that glycosylation was a post-translational modification. They demonstrated that this glycosylated PTHrP was biologically active, in that it exerted a positive contractile effect on cardiomyocytes, with a higher potency than synthetic PTHrP (1-34).

Smaller bands, of appropriate size for amino-terminal and mid-fragment daughter species, were not detected on the western blots of the cell lysates. This may be because the smaller peptides were degraded during processing of the whole cell lysates, or because cleavage of mature PTHrP by members of the prohormone convertase family into daughter peptides does not occur in these tumour cells.

Endoprotease cleavage of mature PTHrP may not occur in pancreatic adenocarcinoma tumour cells either to the absence of specific endoproteases in these de-differentiated cells, or because during PTHrP translation, amino acid substitution occurs one at of the cleavage sites, for example the monobasic $Arg^{37}$, which is then not recognised by the endoprotease.

The 60 kDa protein bands obtained with the anti-PTH1R antibody are an appropriate size for non-glycosylated PTH1R. The 80 kDa band observed in the control cell line PC3 is an appropriate size for the glycosylated PTH1R, [cxliii] and has been obtained by other groups using this antibody.[cxvi] The 160 kDa band is likely to represent a dimer of the glycosylated receptor. A 45 kDa band has also been described using an anti-PTH1R antibody raised against a peptide sequence in the extracellular N-terminal region of the receptor,[cxli] and may represent a truncated form of the receptor.

The dual expression of PTHrP and PTH1R in human pancreatic tumour cell lines, and in tumour resection specimens, and the significant positive correlation between the intensity of the staining for PTHrP and for PTH1R suggests that an autocrine and/or paracrine loop may exist for PTHrP, acting via the PTH1R, in these tumours. Moreover, positive nuclear staining for PTHrP and PTH1R was seen 1-5% of BxPC3 cells, and in tumour cells in some of the resection specimens. PTHrP staining was observed principally in a diffuse nuclear pattern, as observed by other groups.[lxi, lxxvii, lxxx] This suggests that either nucleolar translocation of amino-terminal PTHrP occurs in these cells, possibly mediated by PTH1R, or that amino-terminal PTHrP binds to intranuclear PTH1R.

Absence of hypercalcaemia in these patients with PTHrP-expressing pancreatic adenocarcinomas is similar to the situation seen in patients with prostatic adenocarcinoma, but contrasts to breast adenocarcinoma where hypercalcaemia is common. Possible explanations for this may be that PTHrP is produced and released in insufficient quantities to cause hypercalcaemia, or that PTHrP expressed by these tumours is degraded within the pancreas which is rich in enzymes, or alternatively that PTHrP is processed into peptides that do not cause hypercalcaemia.

Although the function of PTHrP in pancreatic cancer is unknown, it is known to regulate growth in other tumour types, and Bouvet et al have demonstrated that exogenous PTHrP (1-34) stimulates growth in the human pancreatic cell line AsPC-1. The finding of the expression of PTHrP and its receptor in exocrine pancreatic adenocarcinoma warrants further investigation, particularly if PTHrP is confirmed to have growth-regulating effects.

# Chapter 3 EXPRESSION OF PARATHYROID HORMONE-RELATED PEPTIDE AND PARATHYROID HORMONE TYPE 1 RECEPTOR IN NEUROENDOCRINE TUMOURS

## 3.1 Introduction

Neuroendocrine tumours are a relatively rare family of tumours that occur in tissues that contain cells derived from the embryonic crest, neuroectoderm, and endoderm.[cxliv] Thus they may occur at many sites throughout the body, although the majority occur within the gastro-entero-pancreatic axis. The histological diagnosis of neuroendocrine tumours relies first on the identification of general markers of neuroendocrine differentiation, and then on cell-specific characterisation. Neuroendocrine differentiation is evaluated by immunohistochemistry using antibodies against secretory granule proteins (chromogranin A, synaptophysin) and cytosolic proteins (neuron-specific enolase, protein gene product 9.5). The cell-specific characterization of neuroendocrine tumours requires hormone immunohistochemistry.[cxlv]

Neuroendocrine tumours are generally considered to comprise of two main tumour groups: pancreatic endocrine or islet cell tumours and carcinoid tumours. Pancreatic endocrine tumours may be further subclassified and named according to the hormone that they secrete, and consequently produce an associated clinical syndrome, for example, gastrinoma manifesting with the characteristic Zollinger-Ellison syndrome, which is caused by hypergastrinaemia associated with hypersecretion of gastric acid; or insulinoma characterised by hypersecretion of insulin and hypoglycaemia. Carcinoid tumours are traditionally considered to be serotonin (5 hydroxytrytophan) secreting, but frequently produce many other hormone products, including kinins, prostaglandins, substance P, gastrin, somatostatin, corticotrophin, and chromogranin A.[cxlvi]

PTHrP-secreting pancreatic endocrine tumours associated with hypercalcaemia are becoming increasingly recognised as a potential subgroup of pancreatic endocrine tumours.[cxlv] Pancreatic endocrine tumours not associated with hypercalcaemia have also been shown to produce PTHrP. Drucker et al reported a survey of 15 human islet cell tumours not associated with HHM, and found that 13 (87%) stained for PTHrP.[cvi] Carcinoid tumours however are infrequently associated with hypercalcaemia, although HHM has been reported.[cxlvii,cxlviii] There are no reports in the literature of immunohistochemical expression of PTHrP or PTH1R in carcinoid tumours.

The purpose of these experiments was: 1) to investigate by immunohistochemistry the expression of PTHrP and PTH1R in resection specimens from normocalcaemic patients with a variety of neuroendocrine tumour types; 2) to investigate PTHrP and PTH1R expression in neuroendocrine tumour cell lines using immunohistochemistry and western immunoblotting; and 3) to assess whether there is a relationship between PTHrP/PTH1R expression and cellular proliferation as assessed by Ki67 expression.

## 3.2 Material and Methods

### 3.2.a Patient Tissue Specimens

Forty-seven patients with well-characterised neuroendocrine tumour who were undergoing treatment at the Royal Free Hospital were selected. Only the patients whose paraffin-embedded tissues from resection specimens or biopsies were available were included in the study. The following clinicopathological details about the tumour case were collected: histological evidence of vascular invasion, local invasion, and perineural invasion, and the presence of tumour metastases to bone, liver or other organs (Table 5).

All of the patients had been normocalcaemic prior to their procedures. All patients had given consent for the use of the tissue for research, under ethical approval by the Royal Free Hospital ethics committee. Sequential sections (5μm thick) were cut with a microtome from the formalin-fixed, paraffin-embedded tissues, mounted on APES-coated slides, and dried overnight at 60°C.

*Table 5 Patient population and histological details of neuroendocrine tumour resection specimens studied.*

| Case No. | Sex | Age (years) | Description of Resection/Biopsy Specimen | Tumour Invasion | | | Primary Tumour | Other metastases | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Local | Blood vessels | nerves | | Bone | Other Organs |
| 1 | M | 59 | lymph node resection | yes | no | no | appendiceal carcinoid | no | liver, LN |
| 2 | F | 16 | duodenum, jejunum | yes | no | yes | ileal carcinoid | yes | liver, lung |
| 3 | M | 73 | biopsy of paraaortic/ adrenal mass | N/A | N/A | N/A | unknown primary | yes | liver, paraaortic LN |
| 4 | F | 74 | liver nodule | N/A | N/A | N/A | PET | yes | liver |
| 5 | F | 31 | gastric tumour | yes | no | no | ileal carcinoid | yes | liver |
| 6 | F | 54 | lung tumour | yes | no | no | bronchial carcinoid | no | none |
| 7 | F | 46 | hemicolectomy specimen | yes | yes | yes | appendiceal carcinoid | no | liver, LN |
| 8 | F | 47 | liver nodule | no | no | no | gastric paraganglioma | no | liver |
| 9 | F | 28 | appendiceal tumour | yes | no | no | appendiceal carcinoid | no | none |
| 10 | F | 31 | liver metastasis | N/A | N/A | N/A | ampullary PET | no | liver |
| 11 | M | 35 | biopsy of liver metastasis | yes | N/A | N/A | PET | yes | liver |
| 12 | M | 74 | liver nodule | yes | yes | yes | pancreatoduodenal PET | yes | soft tissue |
| 13 | M | 81 | ileal tumour | yes | no | yes | ileal carcinoid | no | LN |
| 14 | F | 74 | neck lymph node | yes | no | no | unknown primary | no | liver, LN |
| 15 | F | 35 | thymic carcinoma | yes | yes | no | thymic carcinoma | no | liver, lungs, local |
| 16 | M | 56 | small bowel tumour | yes | yes | no | ileal carcinoid | no | liver, LN |
| 17 | F | | appendiceal tumour | yes | no | no | appendiceal carcinoid | no | none |
| 18 | M | 55 | pancreatic tumour | no | no | no | insulinoma | no | none |
| 19 | M | 56 | liver nodule | yes | yes | yes | ileal carcinoid | yes | LN, Liver |
| 20 | F | 46 | gastric biopsy | N/A | N/A | N/A | gastric carcinoid | no | liver |
| 21 | M | 43 | bone marrow aspirate | N/A | N/A | N/A | PET | yes | liver, skin |
| 22 | F | 48 | pancreatic tumour | yes | no | no | PET | no | liver, spleen, LN |
| 23 | F | 70 | mesenteric mass | no | no | no | unknown primary | no | LN |
| 24 | F | 43 | pancreatic tumour | yes | yes | no | pancreatic carcinoid | no | liver |
| 25 | M | 43 | appendix | yes | no | no | appendiceal carcinoid | no | none |
| 26 | M | 80 | duodenal biopsy | N/A | N/A | N/A | duodenal carcinoid | no | none |
| 27 | M | 79 | neck lymph node | no | no | no | medullary thyroid cancer | no | LN, local |
| 28 | F | 59 | ileal tumour | yes | yes | yes | ileal carcinoid | no | LN |
| 29 | M | 65 | biopsy of liver metastasis | N/A | N/A | N/A | ileal carcinoid | no | liver |
| 30 | M | 58 | sigmoid colon tumour | yes | yes | no | sigmoid carcinoid | no | none |
| 31 | M | 42 | mesenteric tumour nodule | yes | no | no | paraganglioma | yes | liver, lung, LN |
| 32 | M | 58 | small bowel tumour | yes | yes | no | ileal carcinoid | no | liver, LN |
| 33 | M | 45 | pancreatic tumour | yes | no | no | PET | no | liver |
| 34 | F | 41 | duodenal tumour | yes | yes | no | ampullary PET | no | S.I, liver, LN |
| 35 | F | 56 | pancreatic biospy | no | yes | no | insulinoma | no | none |
| 36 | M | 33 | biopsy of liver metastasis | yes | no | no | PET | no | liver |
| 37 | F | 49 | pancreatic tumour | no | no | no | PET | no | none |
| 38 | M | 70 | gastric biopsy | yes | N/A | N/A | gastric carcinoid | no | none |
| 39 | M | 44 | ileal biopsy | N/A | N/A | N/A | ileal carcinoid | no | Liver |
| 40 | F | 66 | neck tumour | no | no | no | paraganglioma | yes | Liver, LN |
| 41 | M | 43 | biopsy of rectal tumour | yes | no | no | rectal carcinoid | no | liver |
| 42 | M | 66 | biopsy of brain tumour | yes | no | no | unknown primary | no | unknown |
| 43 | M | 36 | liver biopsy | N/A | N/A | N/A | PET | no | liver, LN |
| 44 | F | 56 | tumour in duodenum/head of pancreas | yes | yes | yes | PET | no | liver |
| 45 | F | 53 | liver biopsy | yes | no | no | PET | no | liver |
| 46 | M | 75 | gastric biopsy | yes | N/A | N/A | gastric carcinoid | no | no |
| 47 | M | 76 | small bowel mass resection | No | yes | no | colonic carcinoid | no | LN, local |

**Legend:** F = female, M = male, PET = pancreatic endocrine tumour, LN = lymph node

### 3.2.b Cell Lines

The following neuroendocrine cell lines were studied: BON (human endocrine pancreatic tumour), CRI-G1 (rat NEDH islet cell tumour), NCI-H727 (human bronchial carcinoid), and RIN 5F (rat islet cell tumour). The BON cell line was a gift from Professor CM Townsend (Department of Surgery, University of Texas, USA). The other cell lines were obtained from the European Collection of Animal Cell Cultures. To culture the BON cells, a 1:1 mixture of F12K nutrient mixture (Gibco$^{TM}$, Invitrogen Ltd, Paisley, UK) and DMEM (Sigma) supplemented with 10% FCS (Sigma) was used. The CRI-G1 cells were cultured in DMEM containing 10% FCS and 2mM L-glutamine. The NCI-H727 and RIN 5F cells were cultured in RPMI-1640 medium (Sigma) containing 10% FCS and 2mM glutamine. The human prostate carcinoma cell line PC3 was used as a positive control. All cells were grown at 37°C in a humidified 95% air - 5% $CO_2$ atmosphere.

### 3.2.c Immunohistochemistry on Patient Tissue Sections

*3.2.c.1 Anti-PTHrP and Anti-PTH1R*

Sequential sections were tested with the same antibodies (anti-PTHrP, anti-PTH1R), by the same method as described in Chapter 2. Sections were examined by light microscopy and scored by two independent observers using the previously described method.

*3.2.c.2 Anti-Human Ki-67 Antigen*

The Ki-67 antigen is a nuclear protein, which is defined by its reactivity with monoclonal antibody from the Ki-67 clone.[cxlix] It is preferentially expressed in cell nuclei during all active phases of the cell cycle ($G_1$, S, $G_2$ and M-phases), but is absent in quiescent or resting cells ($G_0$). Monoclonal mouse anti-human Ki-67 (clone MIB-1) (Dako) was optimised on paraffin sections from human tonsil.[cl] Antigenic unmasking was carried out by pressure cooking the slides at 115 °C in 0.1M citrate buffer, pH 6.0 for 135 seconds. A dilution of 1 in 100 was chosen for subsequent experiments. All experiments were performed at room temperature, and were validated by the inclusion of negative (prepared by omission of the primary antibody) and positive (tonsil) control sections. A standard three-step immunoperoxidase

technique with diaminobenzidine as a chromogen (ABC) was used for detection of the Ki67 antigen (Appendix III).

*3.2.c.3 Evaluation of Ki67 staining*

Tumour nuclei showing distinct immunostaining were counted as positive. The percentage of positively staining tumour cell nuclei was estimated from four representative x20 magnification fields. For statistical analyses, percentages were grouped as being: 1 = <1%, 2 = 1-5%, 3 = 6-10%, 4 = 11-30%, 5 = 31-50%, 6 = >50%.

Statistical analyses were performed using the Spearman rank correlation test, and the Mann-Whitney U non-parametric test for group differences as previously described in Chapter 2.2.e.

### 3.2.d Immunohistochemistry on Cultured Cells

Cells were seeded onto four-well slides (Beckton Dickenson, NJ, USA), and grown to approximately 80% confluency. Immunohistochemical expression of PTHrP and PTH1R was assessed using the APAAP method of detection and Fast Red as a chromogen. No pre-treatments were given. After nuclear counterstaining, cells were fixed with 10% normal buffered formaldehyde (NBF), air dried and mounted in Loctite UV (Loctite, UK) adhesive. Experiments were validated by the inclusion of a negative control well (incubated in PBS in replacement of primary antibody), and a positive control well (PC3 cells). For the anti-PTHrP antibody, the specificity of staining was further validated by the inclusion of a well that was incubated with a primary antibody solution of the same concentration of antibody that had been pre-absorbed overnight with excess immunizing peptide. Sections were examined by light microscopy and scored by two independent observers using the method previously described (Chapter 2.2.d).

### 3.2.e Western Immunoblotting

Lysates of the BON, CRI-G1, NCI-H727, RIN 5F cell lines, and the prostatic adenocarcinoma cell line PC3 (chosen as positive control), were prepared by the method outlined in Chapter 2.2.f. Lysate volumes corresponding to 10μg of protein were loaded onto Laemmli SDS-polyacrylamide gels, run and electrotransferred according to the previously described method. Immunoblotting was performed on the membranes using the following antibodies: anti-PTHrP (1-10) (Aphton), at a dilution of 1 in 200; and anti-PTHrP (1-34), anti-PTHrP (38-64), and anti-PTH1R, at the concentrations previously used. For the PTHrP (1-10) and PTHrP (1-34) antibodies, additional positive controls of 15μg of immunizing peptide were included in each run and the results were further validated by incubating parallel blots with the same concentration of antibody that had been pre-absorbed overnight with excess immunizing peptide. Bands were considered to represent PTHrP peptides only if they were abolished or significantly by pre-absorption.

## 3.3 Results

### 3.3.a Immunohistochemistry on Tissue Sections

*3.3.a.1 Expression of PTHrP and PTH1R*

Positive cytoplasmic staining for PTHrP was seen in tumour cells in the majority of neuroendocrine resection specimens studied [38 of 47 (80.9%)] (Figure 13). A relatively higher number of carcinoid tumours than pancreatic endocrine tumours showed positive cytoplasmic staining for PTHrP [22 of 25 (88%) versus 12 of 16 (75%)] (Table 6). Positive cytoplasmic staining for PTH1R was also seen in the majority of the neuroendocrine tumour resection specimens [40 of 47 (85.1%)] (Figure 13). Again, more carcinoids than pancreatic endocrine tumours stained positively [22 of 25 (88%) versus 13 of 16 (81.3%)] (Table 6). Seventy-two percent of tumours showed positive staining for both PTHrP and PTH1R. Variable staining for PTHrP and PTH1R was seen in normal vascular endothelial cells, neurons, some normal epithelial cells, and in adjacent normal pancreatic islets. Preabsorption of the

antibody with the immunizing peptide abolished all staining for PTHrP. Staining for PTH1R was abolished by substitution of normal mouse serum for primary antibody. There was a significant positive correlation between the intensity of tumour staining for PTHrP and PTH1R ($r = 0.323$ p = 0.027) across all of the groups. There was no significant difference in the intensity of staining for either antigen in specimens from primary (n = 26) or secondary tumour (n = 21) specimens. There was no significant association between the presence or intensity of staining for either PTHrP or PTH1R and the other clinicopathological characteristics of the tumours (i.e. histological evidence of vascular invasion, local invasion, and neural invasion, and the presence of tumour metastases to bone, liver or other organs).

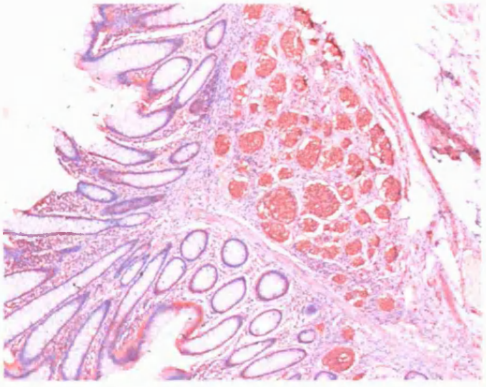
*Table 6 Median tumour cell cytoplasmic immunoreactive product for PTHrP and PTH1R in the neuroendocrine tumour resection specimens and biopsies studied.*

| Type of Tumour | No of Samples | PTHrP positive | Median score (0-9) (range) | PTH1R positive | Median score (0-9) (range) | Both PTHrP and PTH1R positive |
|---|---|---|---|---|---|---|
| **Carcinoid** | 25 | 22 (88%) | 4 (0-6) | 22 (88%) | 3 (0-9) | 20 (80%) |
| **Pancreatic endocrine tumour** | 16 | 12 (75%) | 4 (0-6) | 13 (81.3%) | 3 (0-6) | 11 (68.8%) |
| **Other** | 6 | 4 (66.7%) | 2 (0-4) | 5 (83.3%) | 3 (0-6) | 3 (50%) |

**Legend:** The intensity of staining was graded on a scale from 0 to 3 where, 0 indicated no staining; 1 indicated weak intensity of staining; 2, moderately intense staining; and 3, most intense staining. Sections were also given a score to grade the extent of staining, such that less that 25% positive cells = 1; 25-75% cells = 2; and more than 75% = 3. To obtain a numerical score, the intensity of staining was multiplied by the number of cells stained.

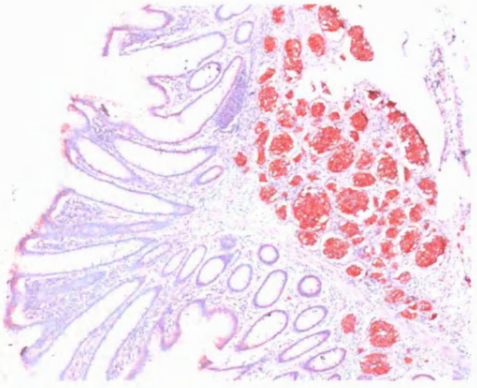
Figure 13 Immunohistochemistry showing positive tumour cell staining for PTHrP (figures a, c, e, & g) and PTH1R (figures b, d, f, & h) in resection specimens of neuroendocrine tumours. Positive staining appears as a red precipitate. Variable staining for both peptide and receptor was seen in normal vascular endothelial cells, neurons, some normal intestinal epithelial cells, and in pancreatic islets.

**Appendiceal Carcinoid (Case No. 25)**



a

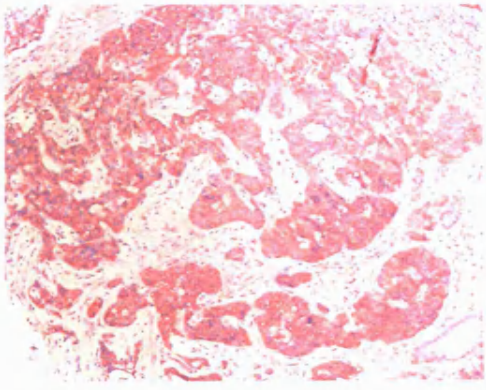
Magnification x25



b

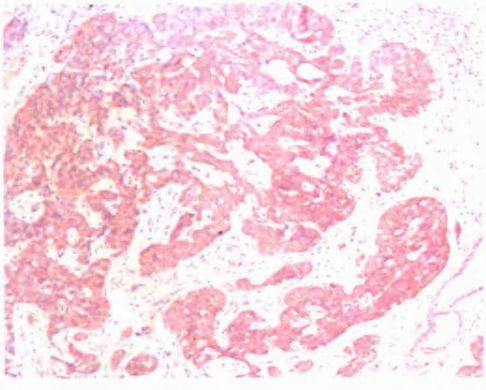
Magnification x25

**Medullary Thyroid Carcinoma – Lymph Node Resection (Case No. 27)**



c

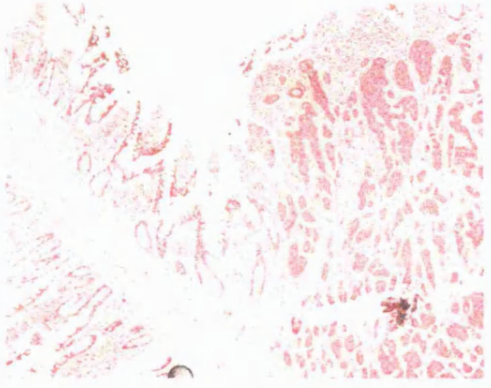
Magnification x50



d

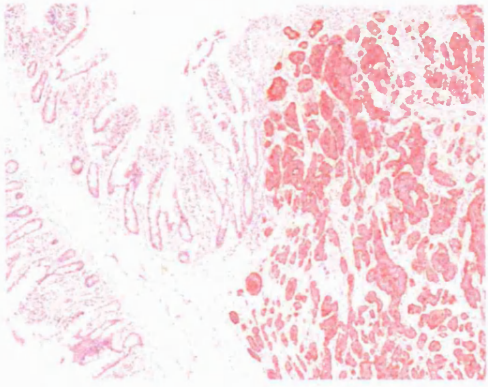
Magnification x50

**Ileal Carcinoid (Case No. 28)**



e

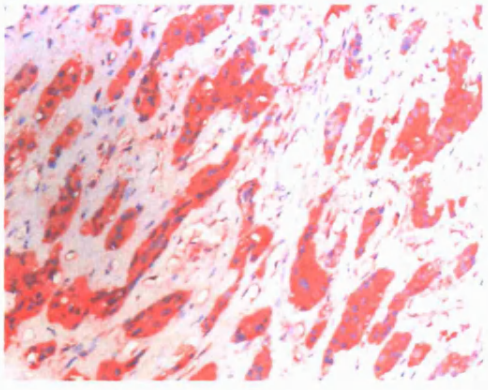
Magnification x25



f

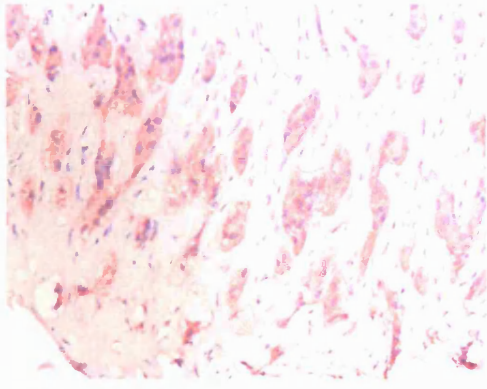
Magnification x25

**Pancreatic Endocrine Tumour – Liver Biopsy (Case No. 43)**



g

Magnification x100



h

Magnification x100

*3.3.a.2 Expression of Ki67*

There was a significant difference in Ki67 staining in primary and secondary tumours ( $p = 0.034$), the Ki67 score being higher in secondary tumours (median percentage cells positive 5-10%) compared to primary tumours (median percentage cells positive 1-5%). Statistical analysis showed a significant negative correlation between the expression of PTHrP and the Ki67 score ($r = -0.378$, $p = 0.016$).

### 3.3.b Immunohistochemistry on Cells

There was variable positive cytoplasmic staining for PTHrP and PTH1R in all 4 cell lines tested (Table 7). There was also nuclear staining for PTHrP in the CRI-G1 cell line in approximately 15% of the cells (Figure 14). Staining for PTHrP and PTH1R was abolished by preabsorption of the PTHrP antibody with immunizing peptide, and by substitution of normal mouse serum for PTH1R antibody, respectively.

*Table 7 Immunohistochemical expression and localisation of PTHrP and PTH1R in the neuroendocrine cell lines studied.*

| Cell line | Intensity and location of immunostaining for PTHrP | Intensity and location of immunostaining for PTH1R |
|---|---|---|
| BON | 9 (C) | 6 (C) |
| CRI-G1 | 9 (C & N) | 6 (C) |
| NCI-H727 | 3 (C) | 6 (C) |
| RIN 5F | 6 (C) | 3 (C) |

**Legend:** C = cytoplasmic, N = nuclear, scoring as described in table 6.

Figure 14 Immunohistochemistry showing positive staining for both PTHrP and PTH1R in rat islet cell tumour cell line CRI G1.
Figure (a) shows cytoplasmic and nuclear staining for PTHrP on cultured CRI G1 cells. The arrows point to positive nuclear staining. (Original magnification x100)

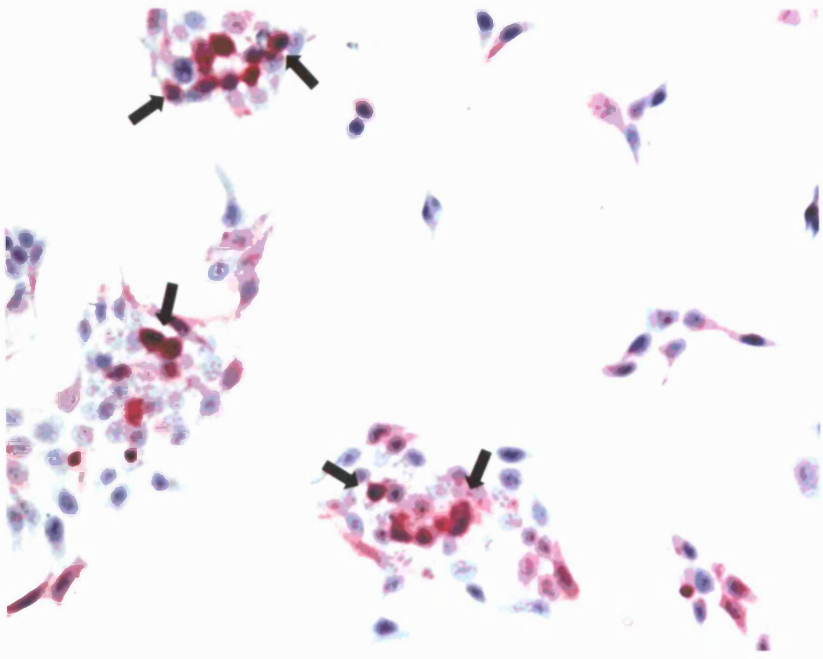


Figure 14(a)

Figure (b) shows cytoplasmic staining for PTH1R on cultured CRI G1 cells. (Original magnification x100)

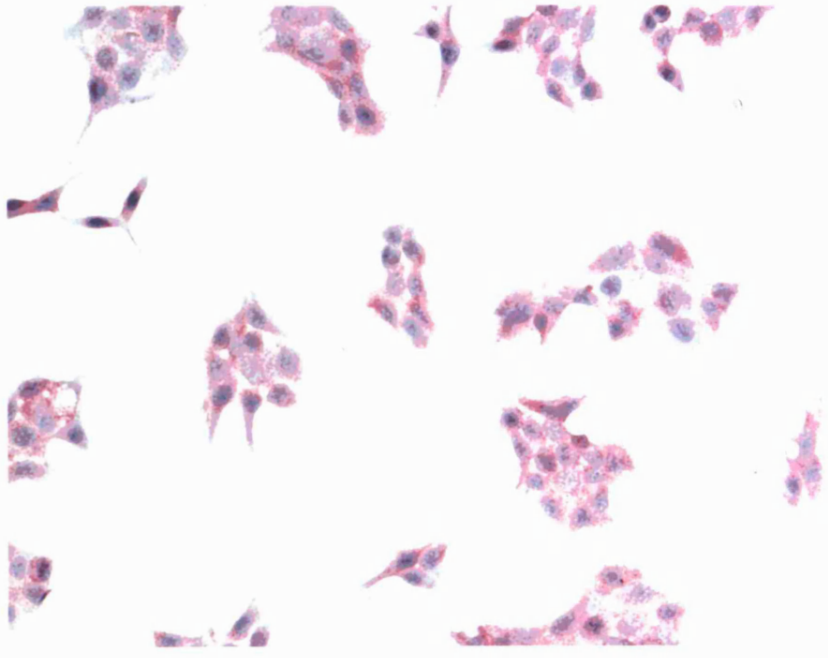


Figure 14(b)

### 3.3.c Western Immunoblotting

*PTHrP (1-10)*

Bands that were almost completely abolished by preabsorption were seen at 30, 35 and 44 kDa in all cell line lysates. An additional band at 15 kDa was observed in the BON, CRI-G1, and NCI-H727 cell lysates (figure 15a), and in the control cell line PC3.

*PTHrP (1-34)*

A distinct band that was completely abolished by preabsorption was present for PTHrP (1-34) at approximately 5 kDa. A single band at 35 kDa that was abolished by pre-absorption was present in all cell line lysates. An additional band at 17 kDa was seen in BON, CRI-G1, and PC3 lysates, and at 30 kDa in the RIN 5F lysate.

*PTHrP (38-64)*

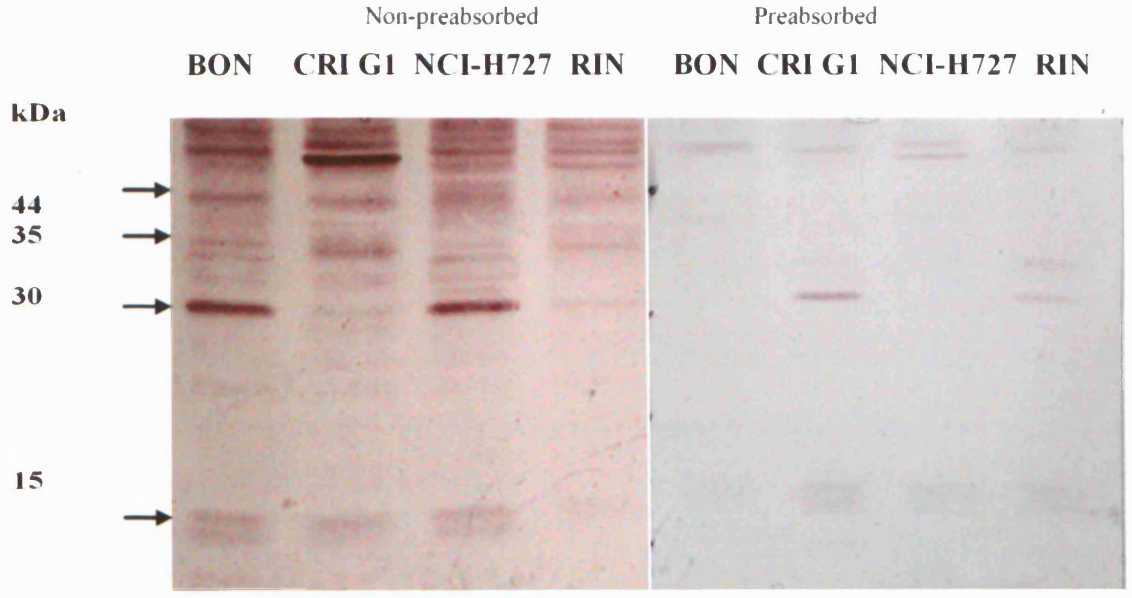
A single band at 35 kDa was observed in lysates from the four neuroendocrine cell lines. A 15 kDa band was observed in RIN 5F and in PC3 cell lysates.

PTH1R

The PTH1R antibody reacted with protein bands at approximately 60, 80 and 160 kDa in all cell lines tested. There was an additional band at 45 kDa in the PC3 lysate (figure 15b).

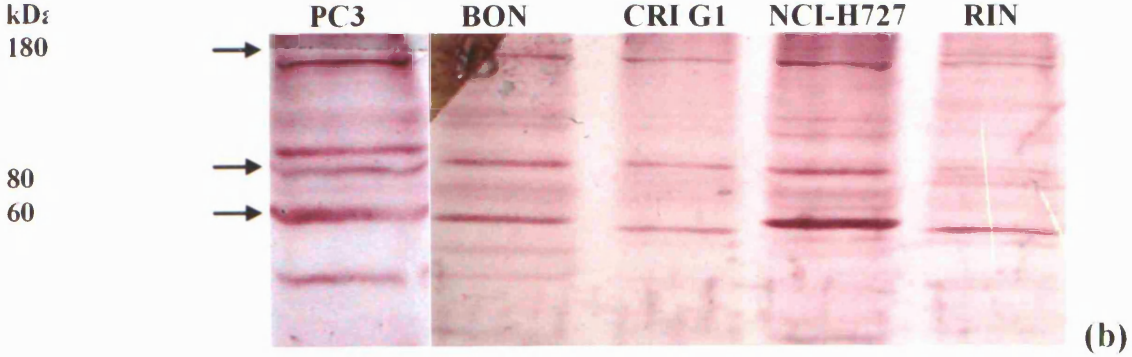
Figure 15 Western immunoblots for PTHrP (1-10) and PTH1R on whole cell lysates from neuroendocrine tumour cell lines.

Figure (a) is a representative immunoblot for PTHrP (1-10). Bands that are attenuated by pre-absorption are seen at 30 kDa, 35 kDa, and 44 kDa in all cell lysates. An additional band at 15 kDa was observed in the BON, CRI-G1, and NCI-H727 cell lysates.




(a)

Figure (b) is a representative immunoblot for PTH1R. Distinct bands are seen at 60 kDa, 80 kDa and 160 kDa in all cell lysates.




(b)

## 3.4 Conclusions

This immunohistochemical study shows that PTHrP and PTH1R protein are highly expressed in both primary and metastatic neuroendocrine tumours, and in neuroendocrine tumour cell lines. PTHrP and PTH1R expression in neuroendocrine tumour cell lines was confirmed on western immunoblotting from whole cell lysates. The bands obtained for PTHrP are similar to those obtained using the same antibodies in pancreatic adenocarcinoma cell line lysates, and are appropriate sizes for PTHrP (1-139) and PTHrP (1-141) molecules and polymerised PTHrP molecules, or possible glycoylated forms of mature PTHrP. Again, smaller bands of sizes, more appropriate for amino-terminal and mid-fragment daughter species, were not detected. The bands obtained for the PTH1R are also appropriate sizes for the non-glycosylated PTH1R, the glycosylated receptor,[cxliii] dimers of the glycosylated receptor, and a truncated form of the receptor.[cxli]

Although generally slow growing, neuroendocrine tumours are often widely metastasised at presentation, and a significant proportion demonstrates aggressive behaviour that may be difficult to manage. Surgery has a potentially curative role in resectable disease and has a role in debulking liver metastases by liver resection. The role of systemic chemotherapy is generally limited to well-differentiated pancreatic endocrine tumours, and alpha interferon is of limited benefit in patients with large volume disease. Somatostatin analogues, while useful for the control of hormone-mediated symptoms, have little in the way of anti-tumour effect. Treatment of liver metastases with loco-regional ablative strategies, such as hepatic-artery embolization and trans-arterial chemoembolization, provide an effective alternative treatment for some hepatic metastases, however these procedures have no impact on extra hepatic disease and carry a significant (up to 6%) mortality rate.

Promising new therapies include receptor-targeted radionuclide therapy, taking advantage of the common expression of somatostatin receptor in neuroendocrine tumours. The concept of this form of targeted therapy is to visualise the tumour with the diagnostic tracer (Indium-111-octreotide) in order to make an estimation of tumour load, and then change the isotope label on the peptide to a higher energy isotope, preferably a β-emitter, in order to target radiotherapy to the tumour cell

('magic bullet'). Recent trials using a number of different labelled somatostatin analogues in patients with therapy resistant and progressive disease have shown promising results.[cli, clii, cliii]

Two problems may occur with somatostatin receptor-targeted therapy, namely that these patients are frequently on concurrent treatment with somatostatin analogues, such as octreotide, in order to control hormonal symptoms, and therefore a proportion of somatostatin receptors may down-regulated or occupied, and unavailable for cellular binding and uptake of a radiolabeled somatostatin analogue. Another problem is that neuroendocrine tumours may 'de-differentiate' and lose the ability to produce a variety of peptides, such as chromogranin A, as well as G-protein-coupled receptors, such as somatostatin receptors. This usually coincides with the development of aggressive widely disseminated disease which is resistant to current existing treatments, in particular somatostatin receptor-targeted therapy which is ineffective in this situation. PTH1R is commonly expressed by these tumours. If PTH1R expression is not lost as these tumours 'de-differentiate', PTH1R-targeted therapies may be a promising new alternative treatment strategy for these difficult to treat tumours. Furthermore, PTH1R has been previously localised to the nucleus, [liii, cliv] as receptor ligands, which may make this receptor a useful portal of entry to the tumour cell nucleus.

The observation that there is a negative correlation between immunoreactivity for PTHrP and the proliferation marker Ki67 can be interpreted in two ways: 1) that PTHrP expressed by neuroendocrine tumour cells inhibits its own growth, and/or 2) that PTHrP expression by neuroendocrine tumour cells is diminished as the tumour cell escapes cell cycle regulatory mechanisms and increases in proliferation. There was no correlation between PTH1R immunoreactivity and Ki67 staining, which was also not significantly different in primary and secondary tumours. If PTH1R expression is not lost as these tumours increase in proliferation and metastasise, PTH1R may further be an attractive target for receptor-targeted therapy.

The co-expression of PTHrP and PTH1R protein in human neuroendocrine tumours, and the positive correlation between the intensity of staining for PTHrP and PTH1R, suggests that an autocrine and/or paracrine loop exists for PTHrP in these tumours.

The detection of PTHrP in the nucleus of the rat islet tumour cell line CRI G1, also suggests that nuclear translocation of PTHrP may occur in these cells.

# Chapter 4 EXPRESSION OF PARATHYROID HORMONE-RELATED PEPTIDE AND THE PARATHYROID HORMONE TYPE 1 RECEPTOR IN HEPATOCELLULAR CARCINOMA

## 4.1 Introduction

The expression of PTHrP and PTH1R by the well differentiated hepatocellular carcinoma cell line HepG2 was described by Li et al in 1996.[clv] However there are few published data relating to the expression of PTHrP in human hepatocellular carcinoma specimens, and to date, none on the expression of PTH1R. This is perhaps surprising, as elevated serum PTHrP causing hypercalcaemia associated with hepatocellular carcinoma has been described,[clvi, clvii] and an association of hypercalcaemia with hepatocellular carcinoma has been known about since Keller first described it in 1965.[clviii]

In contrast, immunoreactivity for PTHrP has been documented by Roskams et al and others in human cholangiocarcinoma resection specimens,[clix, clx] and in human liver cell cultures with a bile duct phenotype.[clxi] Cholangiocarcinoma is well known to be associated with humoral hypercalcaemia of malignancy,[clxii, clxiii, clxiv] and indeed the first case of malignancy-associated hypercalcaemia was reported in 1923 by Zondek et al in a patient with cholangiocarcinoma.[cii]

The aims of these experiments were: 1) to investigate by immunohistochemistry the expression of PTHrP and its corresponding PTH1R in hepatocellular carcinoma tumour resection specimens from patients who were normocalcaemic at the time of resection; and 2) to investigate by immunohistochemistry and western immunoblotting the expression of PTHrP and PTH1R in hepatocellular cancer lines.

## 4.2 Materials and Methods

### 4.2.a Patient Tissue Specimens and Cell Lines

*4.2.a.1 Patient Tissue Specimens*

Twenty cases of well-characterised hepatocellular carcinoma (HCC) were selected from the pathology archives of the Royal Free Hospital from May 1997 to December 2000. The study population was comprised of 20 patients in total, 4 female and 16 male, median age 54 years (range 36-69 years). Sixteen of the cases were from patients who had undergone orthotopic liver transplantation at the Royal Free Hospital for cirrhosis, and 4 were from patients who had undergone segmental hepatic resection for HCC (case numbers 10, 18, 19, 20) (Table 8). All patients had been normocalcaemic pre-operatively, and all had given consent for the use of their liver tissue for research according to a protocol approved by the Royal Free Hospital ethics committee. Liver specimens had been received within one hour after removal, sliced and fixed in formalin for 24 hours. Protocol samples were paraffin processed and tissue sections (H & E and reticulin stains) were prepared using routine histological laboratory standard procedures. Sequential sections (5μm thick) were cut with a microtome from the formalin-fixed, paraffin-embedded tissues, mounted on APES-coated slides, and dried overnight at 60°C.

The following clinicopathological details about the tumour case were collected: tumour size, tumour differentiation, histological evidence of vascular and local invasion, and the presence of multiple hepatocellular tumours.

*4.2.a.2 Cell Lines*

The following tumour cell lines were studied: PLC/PRF/5 (human liver hepatoma), HepG2 (human hepatocyte carcinoma) and MCA RH 7777 (rat buffalo hepatoma). The cell lines were obtained from the European Collection of Animal Cell Cultures. PLC/PRF/5 and HepG2 were grown in DMEM containing 10% FCS and 2mM L-glutamine. MCA RH 7777 was grown in DMEM containing 10% FCS, 4mM L-glutamine, 1% non-essential amino acids (NEAA) (Life Technologies, Paisley, UK), and 1% vitamins (Life Technologies). The human prostate carcinoma cell line PC3

was used as a positive control. All cells were grown at 37°C in a humidified 95% air - 5% $CO_2$ atmosphere.

### 4.2.b Immunohistochemistry

*4.2.b.1 Patient Tissue Sections*

Sections were deparaffinized in xylene, and rehydrated through graded alcohol and distilled water. The sections were tested with anti-PTHrP (1-10) antibody (Aphton) and anti-PTH1R (Lab Vision) at the same concentrations as outlined in Chapter 2. The same methods were employed, with the exception that endogenous avidin-binding activity was blocked by incubating the tissue sections with 0.1% avidin for 15 minutes and then after rinsing, incubating them with 0.01% biotin for 15 minutes (Vector Laboratories Ltd, Peterborough, UK). This additional stage was performed after any pre-treatment, and before incubation with rabbit serum. Sections were examined by light microscopy and scored by two independent observers using the method described in Chapter 2.2.d. Statistical analyses were performed by the previously described methods (Chapter 2.2.e).

*4.2.b.2 Cultured Cells*

Immunohistochemical detection of PTHrP and PTH1R was carried out on cultured cells according to the previously described method (Chapter 3.2.d). Experiments were validated by the inclusion of a negative control well (incubated in PBS in replacement of primary antibody), and a positive control well (PC3 cells). As a competitive control, in another well, cells were incubated with primary anti-PTHrP antibody that had been pre-incubated overnight at 4°C with 0.25mg of immunizing peptide.

Sections were examined by light microscopy and scored by two independent observers using the method previously described in Chapter 2.2.d.

### 4.2.c Western Immunoblotting

Lysates of the following cell lines were prepared by the method outlined in Chapter 2.2.f: PLC/PRF/5, HepG2 and PC3. Volumes of lysate corresponding to 10μg of protein were loaded onto Laemmli SDS-polyacrylamide gels, run and electrotransferred according to the previously described method. Immunoblotting was performed on the membranes using the following antibodies: anti-PTHrP (1-10), anti-PTHrP (1-34), anti-PTHrP (38-64), and anti-PTH1R, at the same concentrations as previously used. For the anti-PTHrP (1-10) and anti-PTHrP (1-34) antibodies, an additional positive control of 15μg of immunizing peptide was included in each run, and results were validated by incubating parallel blots with the same concentration of antibody that had been pre-absorbed overnight with excess immunizing peptide. Bands were considered to represent PTHrP peptides only if they were abolished or significantly attenuated by pre-absorption.

## 4.3 Results

### 4.3.a Immunohistochemistry

*4.3.a.1 Patient Tissue Sections*

All 20 cases of HCC showed positive cytoplasmic tumour cell staining for PTHrP [median score 6 (range 3-6)] (Table 8; Figure 16) and positive cytoplasmic tumour cell staining for PTH1R [median score 4 (range 1-6)] (Table 8; Figure 17). In the majority of the specimens studied there was also generalised weaker staining for PTHrP and PTH1R in the surrounding liver tissue [median scores, 4 (range 0-6) and 3 (range 0-6) respectively]. In normal bile ducts there was strong staining for PTHrP [median score 9 (range 3-9)] and variable staining for PTH1R [median score (range 0-4)]. Pre-absorption of the antibody with the immunizing peptide abolished all staining for PTHrP. All staining for PTH1R was abolished by substitution of normal mouse serum for primary antibody.

*Table 8 Histological details and immunohistological expression of PTHrP and PTH1R in resection specimens from 20 patients with hepatocellular carcinoma.*

| Case No | Age (yrs) | Sex | Tumour differentiation | Tumour size (mm) | Tumour invasion | | Multiple HCC tumours | Aetiology of Liver Disease | Tumour cell staining | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Vascular | Local | | | PTHrP (0-9) | PTH1R (0-9) |
| 1 | 48 | F | moderate | 20 | no | no | no | ALD/AATD | 6 | 4 |
| 2 | 61 | M | moderate | 22 | no | no | no | cryptogenic | 6 | 4 |
| 3 | 58 | M | moderate | 75 | yes | yes | satellite | cryptogenic | 6 | 6 |
| 4 | 54 | M | well | 12 | no | yes | no | HBV/HCV | 6 | 3 |
| 5 | 66 | M | moderate | 59 | yes | yes | yes | AATD | 6 | 3 |
| 6 | 61 | M | well | 110 | no | no | yes | non-cirrhotic | 6 | 3 |
| 7 | 36 | M | moderate | 15 | no | no | yes | HBV | 6 | 4 |
| 8 | 42 | M | moderate | 15 | no | no | yes | HBV/HDV | 6 | 6 |
| 9 | 52 | M | well | 28 | no | no | no | HCV | 6 | 4 |
| 10 | 69 | M | well | 75 | no | no | no | HCV | 4 | 1 |
| 11 | 54 | M | well | 9 | no | no | yes | ALD | 6 | 6 |
| 12 | 50 | M | well | 45 | yes | yes | no | ALD | 6 | 4 |
| 13 | 52 | M | moderate | 29 | no | no | yes | HBV/ HCV | 6 | 4 |
| 14 | 46 | M | well | 45 | no | no | yes | ALD | 3 | 1 |
| 15 | 58 | M | well | 33 | no | no | yes | HBV | 6 | 4 |
| 16 | 51 | F | moderate | 70 | no | no | yes | HCV | 6 | 3 |
| 17 | 57 | M | well | 12 | no | yes | no | viral | 6 | 3 |
| 18 | 55 | M | moderate | 15 | no | no | no | HBV/non-cirrhotic | 6 | 6 |
| 19 | 66 | F | moderate | 55 | no | no | no | non-cirrhotic | 6 | 4 |
| 20 | 68 | F | moderate | 195 | no | no | no | non-cirrhotic | 4 | 4 |

**Legend:** M = male, F = female, HBV = Hepatitis B virus, HCV = Hepatitis C virus, HDV = Hepatitis D virus, ALD = Alcoholic liver disease, AATD = alpha-1 antitrypsin deficiency

The immunoreactive product was scored between 0 and 9. To obtain a numerical score, the intensity of staining (scored from 0 to 3) was multiplied by the number of cells stained (scored between 0 and 3).

There was no significant difference between the tumour cell staining scores for PTHrP or PTH1R and the grade of tumour differentiation (only moderate or well differentiated tumours were included in this series), tumour size (grouped for statistical evaluation into tumours that were < 20mm or ≥ 20mm), histological evidence of vascular invasion, local invasion, and the presence of multiple hepatocellular carcinoma tumours. There was a positive correlation between the tumour cell staining obtained for PTHrP and PTH1R that approached significance ($R = 4.4, p = 0.054$).

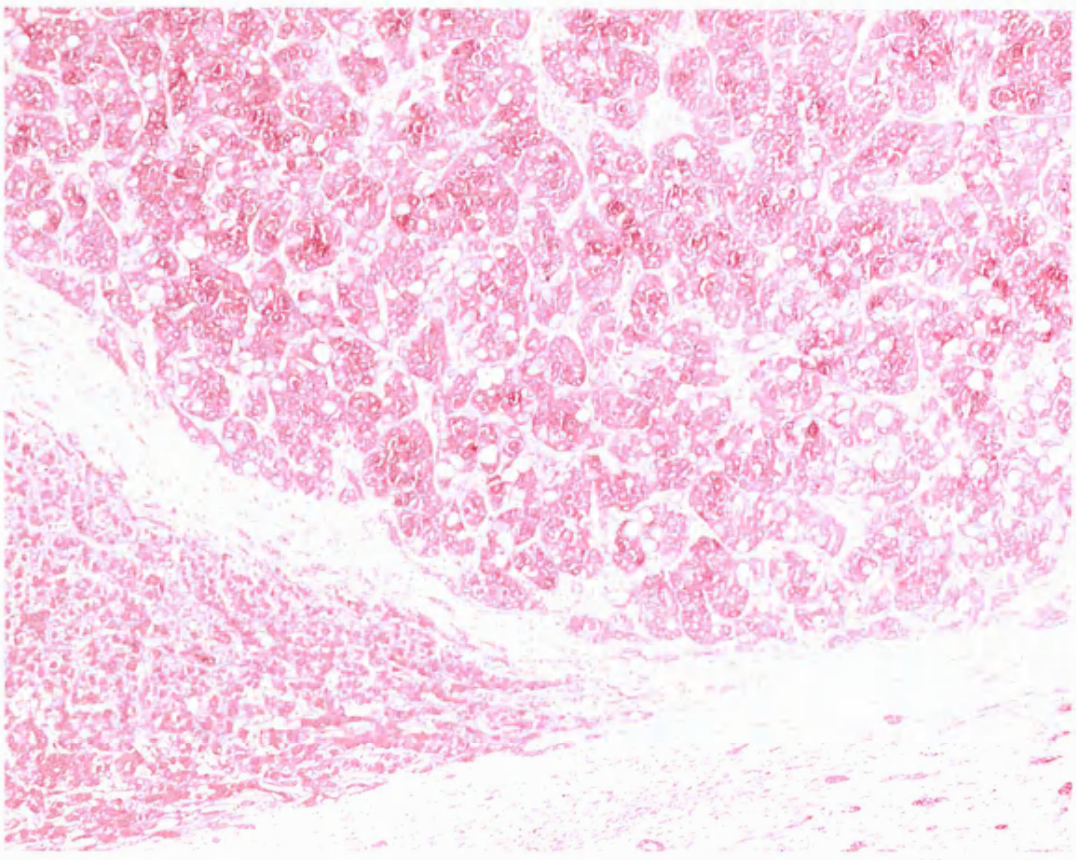


Figure 16(a)

Figure 16 Immunohistochemistry showing positive tumour cell staining for PTHrP in resection specimens of human hepatocellular carcinoma.

The figures are images from three different cases of hepatocellular carcinoma. Positive staining appears as a red precipitate.

Figure (a) shows strong positive cytoplasmic staining for PTHrP in tumour cells and in normal hepatic ducts, with weaker positive cytoplasmic staining in hepatocytes in the surrounding cirrhotic tissue. Figure (b) shows positive cytoplasmic staining for PTHrP in a subset of neoplastic cells within the tumour. Figure (c) shows positive tumour cell staining for PTHrP with strong staining in some tumour cells. (The images are photographed at x100 magnification).

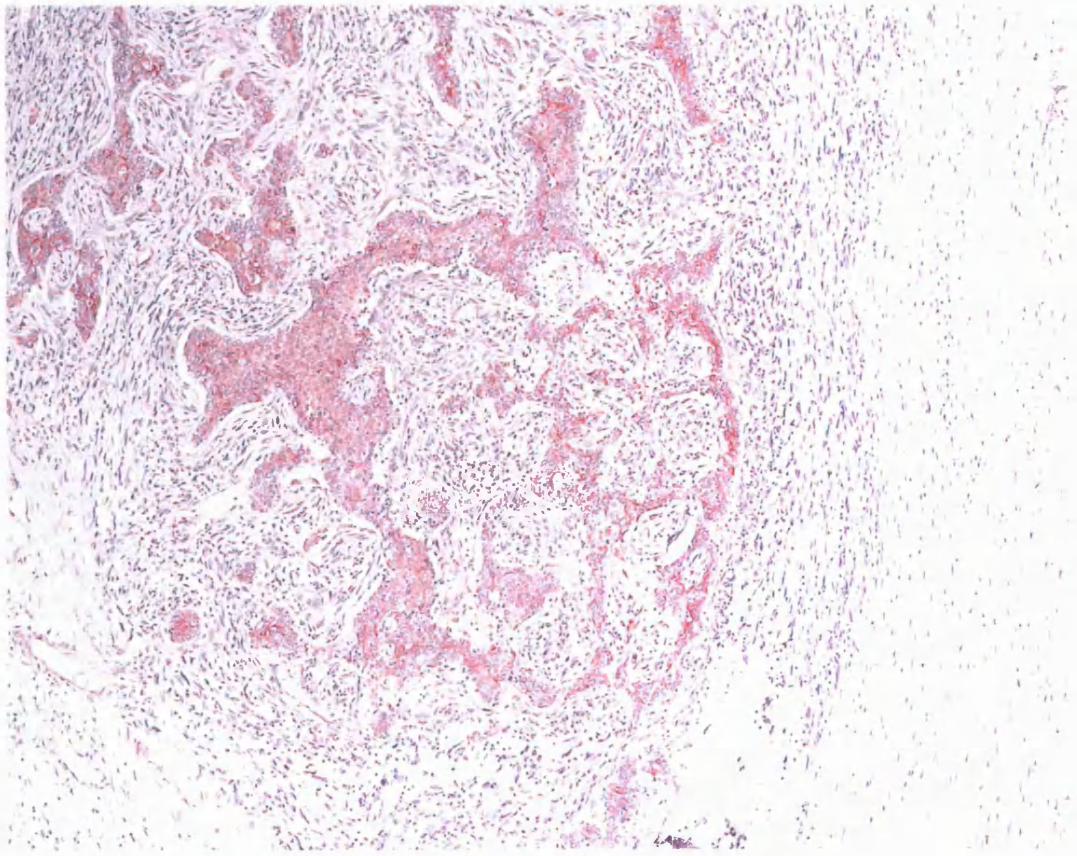


Figure 16 (b)

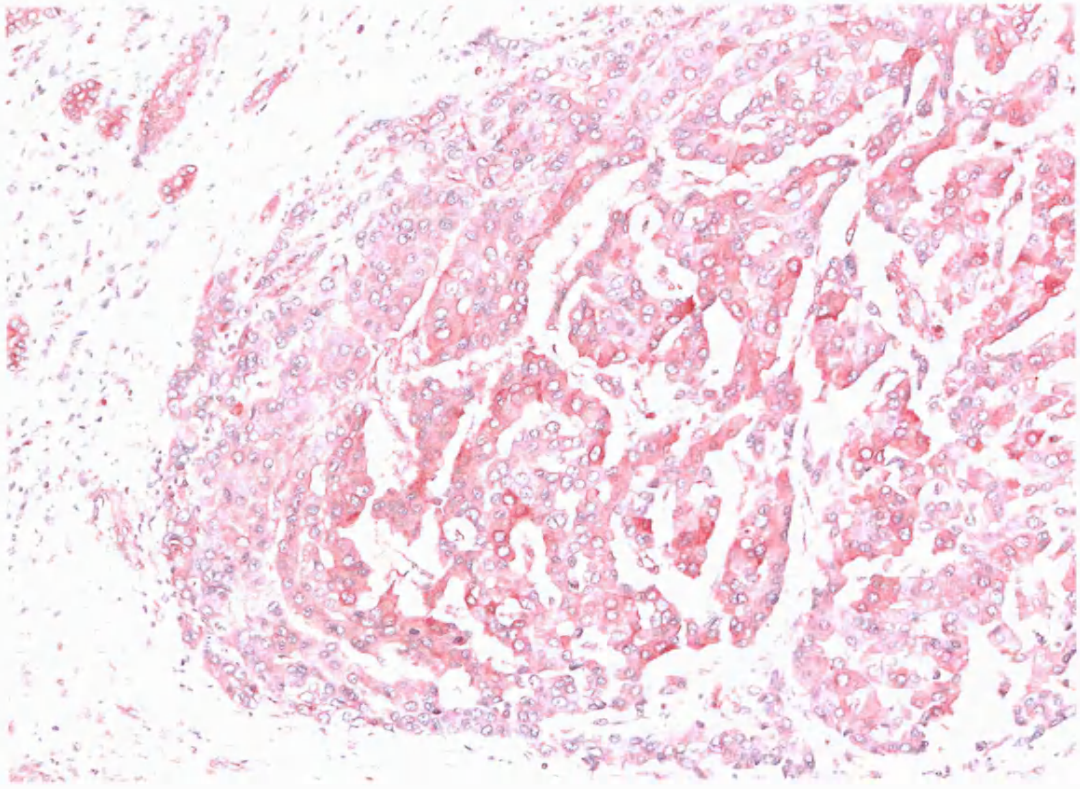


Figure 16 (c)

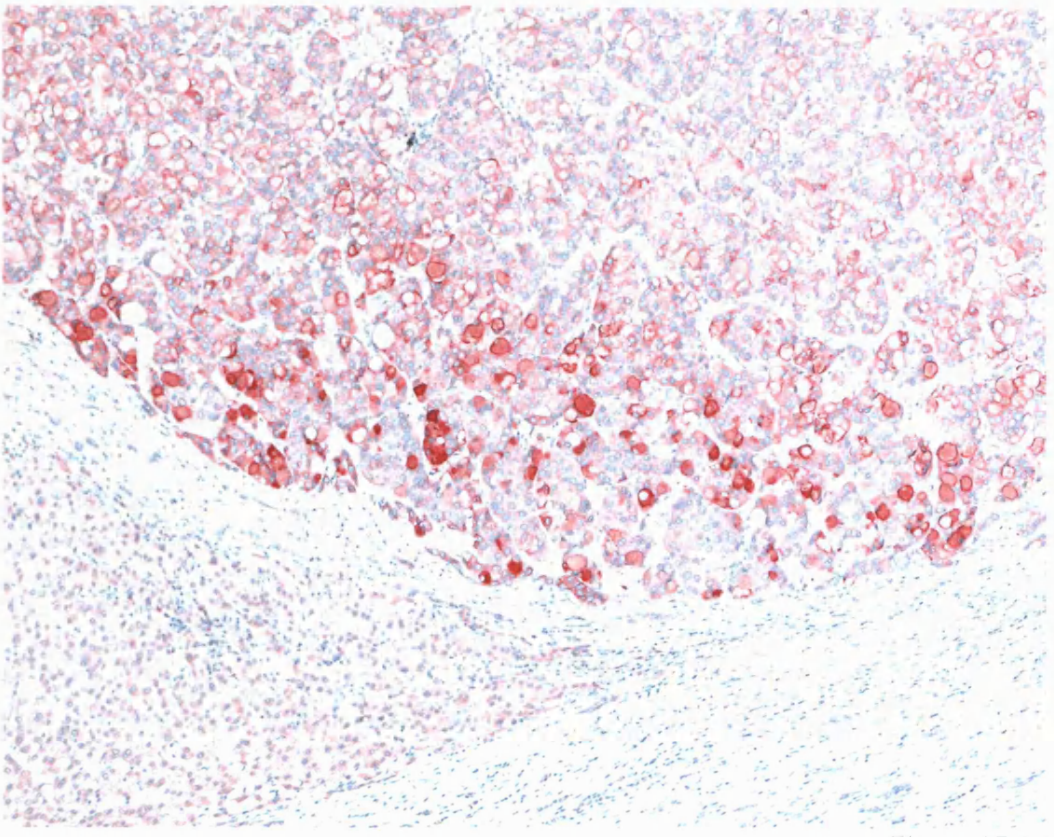


Figure 17 (a)

Figure 17 Immunohistochemistry showing positive tumour cell staining for PTH1R in resection specimens of human hepatocellular carcinoma.

Positive staining appears as a red precipitate. Figures (a) and (b) are images of the same two cases of hepatocellular carcinoma as shown in Figure 16 (a) and (b).

Figure (a) shows strong cytoplasmic staining for PTH1R in some tumour cells, and weak cytoplasmic staining in the surrounding cirrhotic tissue (photographed at magnification x100). Figure (b) shows positive cytoplasmic staining for PTH1R in a subset of neoplastic cells within the tumour (photographed at magnification x100). Figure (c) is a detail of the case in figure (a) (photographed at magnification x 200).

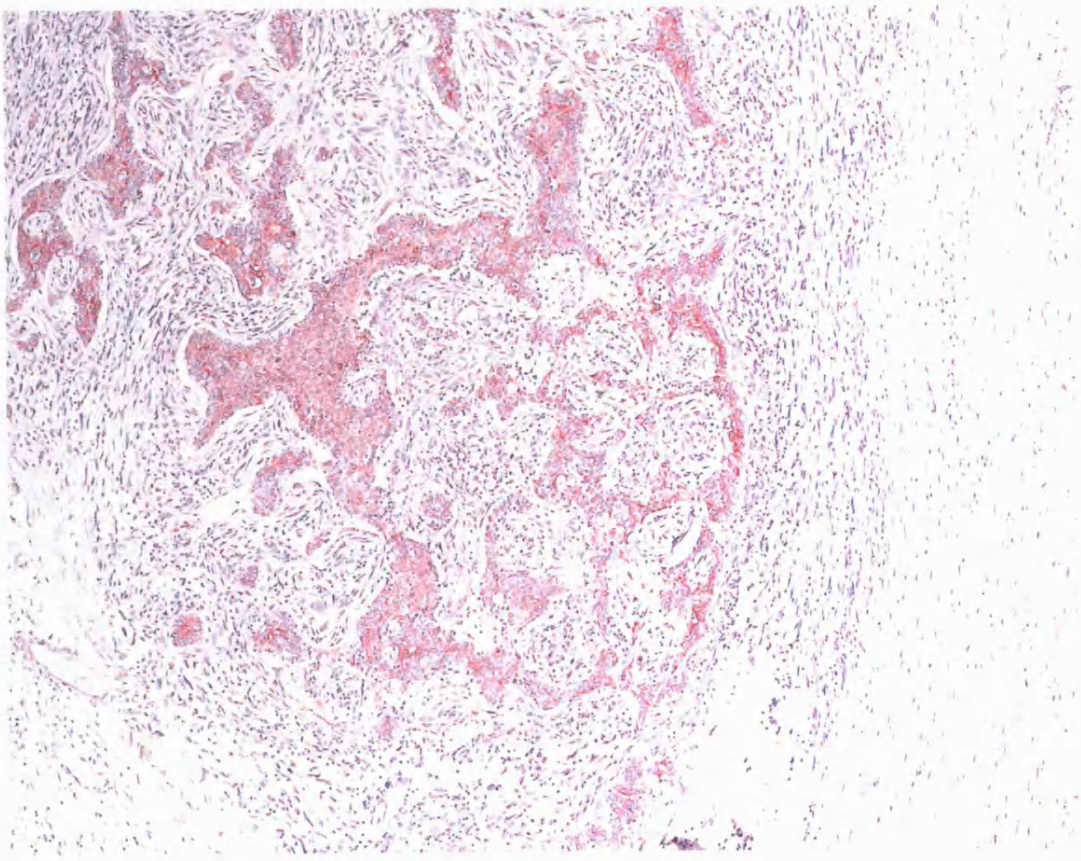


Figure 17 (b)

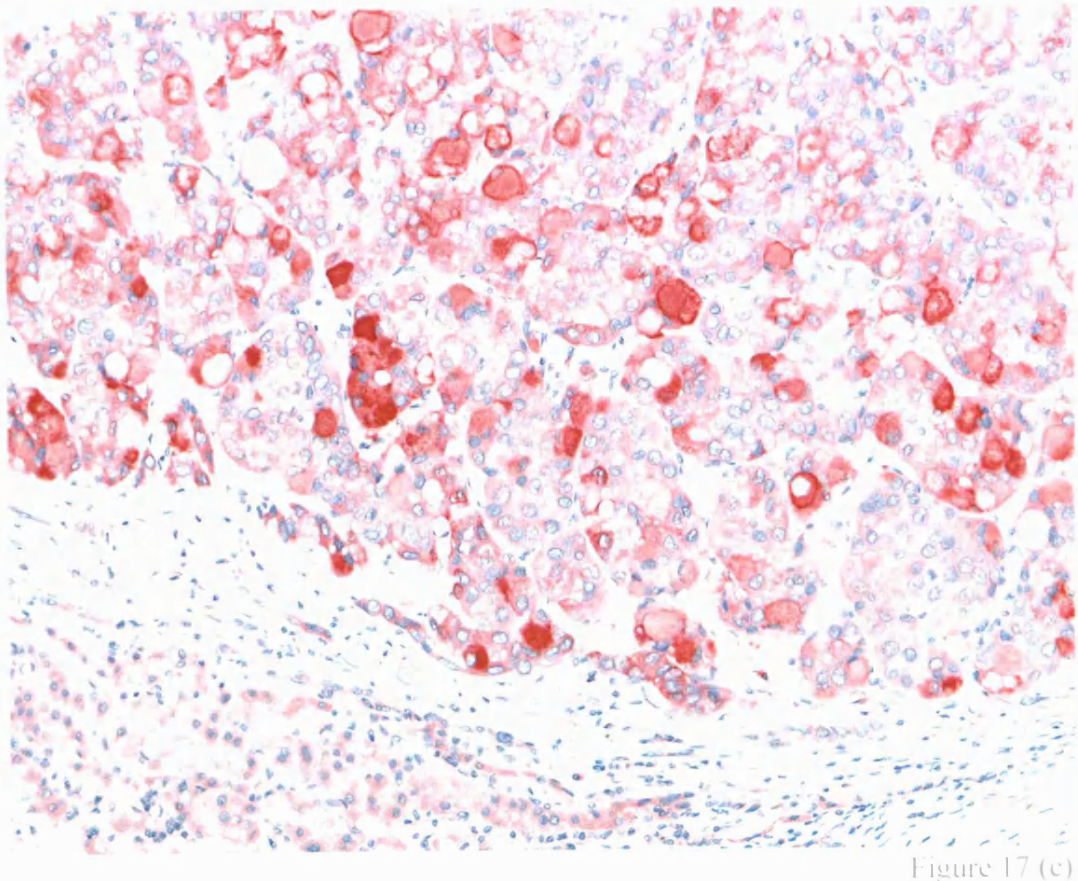


Figure 17 (c)

*4.3.a.2 Cultured Cells*

There was variable positive cytoplasmic staining for PTHrP and PTH1R in all 3 cell lines tested (Table 9; Figure 18). There was also nuclear staining for PTHrP in the MCA RH 7777 cell line in 1-5 % of the cells.

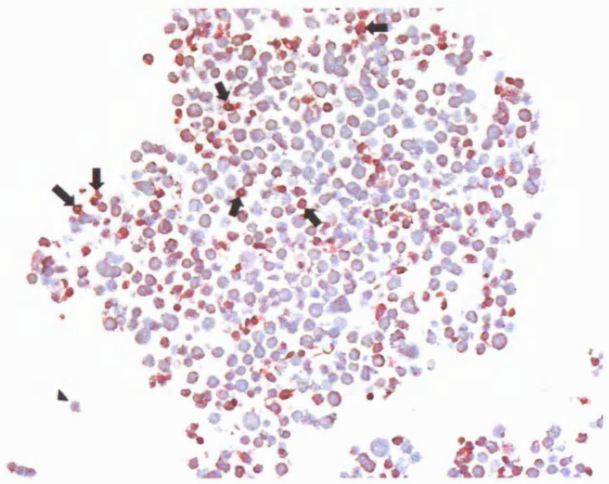
*Table 9 Immunohistochemical localisation of PTHrP and PTH1R in the hepatocellular carcinoma cell lines studied.*

| Cell line | Intensity of staining and localisation of | |
|---|---|---|
| | PTHrP (0-9) | PTH1R (0-9) |
| HepG2 | 3 (C) | 3 (C) |
| MCA RH 7777 | 3 (C & N) | 6 (C) |
| PLC/PRF/5 | 2 (C) | 4 (C) |

**Legend:** C = cytoplasmic, N = nuclear

Figure 18 Immunohistochemistry showing positive staining for both PTHrP and PTH1R in rat hepatocellular carcinoma cell line MCA RH 7777.

Figure 18a shows cytoplasmic and nuclear staining for PTHrP in cultured MCA RH 7777 cells. The arrows point to positive nuclear staining. (Original magnification x100).



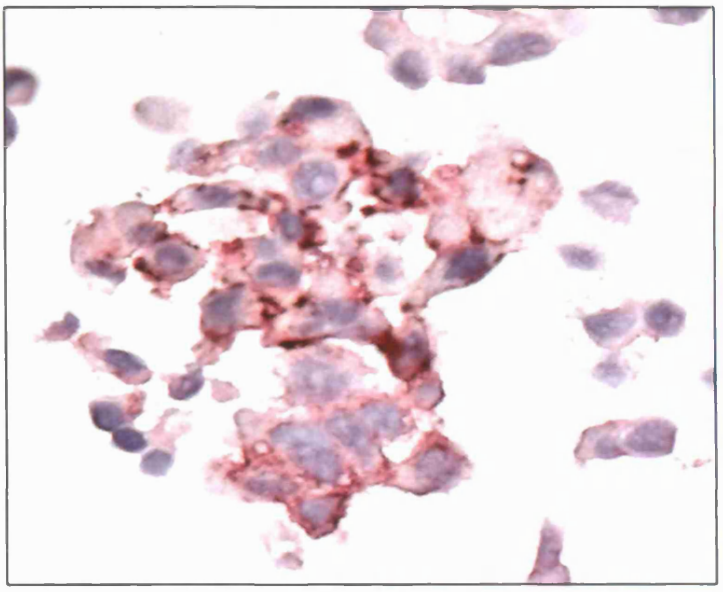


Figure 18b

Figure 18b Immunohistochemistry showing positive cytoplasmic staining for PTH1R in rat hepatocellular carcinoma cell line MCA RH7777 (original magnification x200).

### 4.3.b Western Immunoblotting

*PTHrP (1-10)*

Bands that were almost completely abolished by preabsorption were seen at 15, 30, 35 and 44 kDa in all 3 cell line lysates (figure 19a).

*PTHrP (1-34)*

A distinct band that was completely abolished by preabsorption was present for PTHrP (1-34) at approximately 5kDa. Distinct bands that were completely abolished by preabsorption were seen at 17, 35, and 45 kDa in all 3 cell line lysates.

*PTHrP (38-64)*

Bands at 15, 35, and 50 kDa were observed in HepG2 and PLC/PRF/5 lysates, and at 15- and 50 kDa in the control PC3 lysate.

PTH1R

The PTH1R antibody reacted with protein bands at approximately 60, 80 and 160 kDa in all the cell lines tested. There was an additional band at 45 kDa in the PC3 lysate figure 19b.

Figure 19 Western immunoblots for PTHrP (1-10) and PTH1R on whole cell lysates from hepatocellular carcinoma cell lines.

Figure (a) is a representative immunoblot for PTHrP (1-10). Bands that are attenuated by pre-absorption are seen at 17 kDa, 30 kDa, 35 kDa, and 44 kDa in all cell lysates.

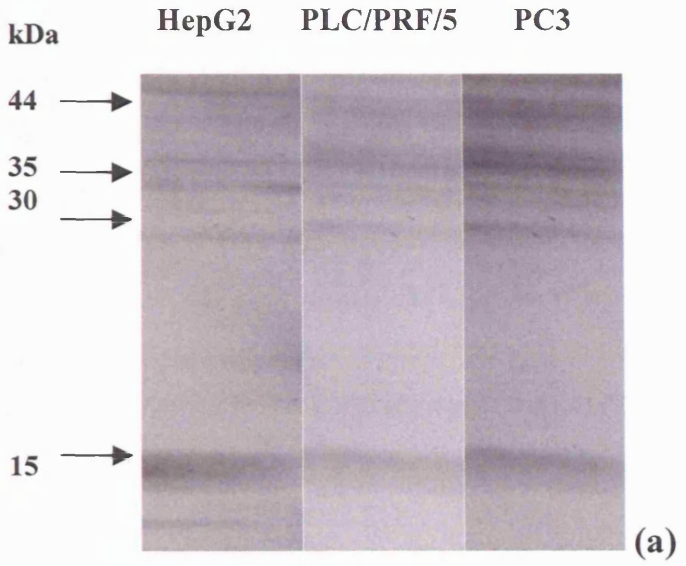
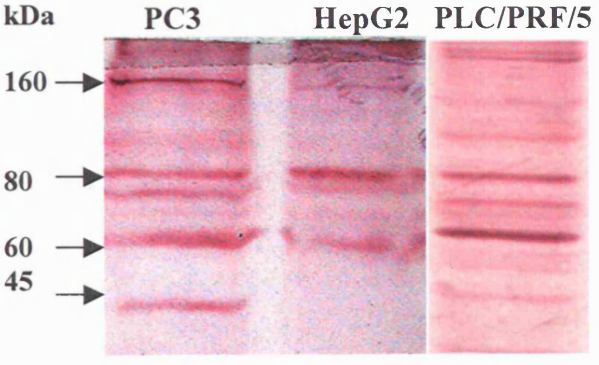



Figure (b) is a representative immunoblot for PTH1R. Distinct bands are seen at 60 kDa, 80 kDa and 160 kDa in all cell lysates.



## 4.4 Conclusions

These results show that PTHrP and PTH1R protein are strongly expressed in the cytoplasm of tumour cells in human HCC resection specimens and by HCC cell lines. PTHrP and PTH1R expression in cell lines was confirmed on western immunoblotting from whole cell lysates. Bands obtained for PTHrP and PTH1R were comparable to those previously obtained using these antibodies on cell lysates from pancreatic cancer and neuroendocrine tumour cell lines. The bands obtained for PTHrP are appropriate sizes for PTHrP (1-139) and PTHrP (1-141) molecules and polymerised PTHrP molecules, or possible glycosylated forms of mature PTHrP. The bands obtained for PTH1R are also appropriate sizes for the glycosylated receptor, dimers of the glycosylated receptor, and a truncated form of the receptor. The 60 kDa protein band detected by the anti-PTH1R antibody may represent the non-glycosylated form of the PTH1R which is fully functional and has an estimated molecular weight of 60 kDa.[clxv, cxli, cxliii]

Hepatocellular carcinoma ranks fifth in frequency among world-wide malignancies, and is on the increase in many countries, particularly in areas where hepatitis C virus is more common than hepatitis B virus infection.[clxvi, clxvii, clxviii] Indeed, mortality from all causes of primary liver cell cancer has almost doubled in 15 years in the UK. The prognosis of this cancer remains dismal, with an overall 5-year survival rate world-wide of only 2% because of late diagnosis. [clxix] Alpha-fetoprotein (AFP) is currently the most important tumour marker for the diagnosis of HCC. However, as a serum tumour marker, AFP lacks sensitivity, as a considerable proportion of HCCs do not produce AFP, or elevate AFP serum level only minimally. Thirty-five percent of HCCs smaller than 3cm do not produce an elevated AFP level [clxix] which makes early diagnosis difficult with this marker alone. AFP is also elevated, sometimes to equivocally high levels (20-400 ng/ml), in serum from cirrhotic patients in whom the majority of HCCs occur. If serum levels of PTHrP, which are undetectable in normal individuals, are found to be elevated in patients with HCC, PTHrP may be a useful tumour marker for HCC, particularly as it would be released by tumours directly into the systemic, rather than the portal circulation.

A study measuring serum levels of PTHrP in patients with HCC is therefore warranted. Assays used for PTHrP detection in serum would have to be sufficiently sensitive to detect low levels of circulating PTHrP, as it seems likely that if detected, the levels would not be as high as those observed in patients with hypercalcaemia of malignancy, as most patients with HCC do not have hypercalcaemia. Additionally, any study measuring serum levels of PTHrP in patients with HCC would have to incorporate cirrhotic individuals with no evidence of HCC, as PTHrP was found to be expressed in background cirrhotic liver, and so may also be detectable in the serum of cirrhotic patients.

Both PTHrP and PTH1R were strongly expressed by human HCC tumour cells. The intensity of staining for both PTHrP and PTH1R positively correlated, suggesting that an autocrine and/or paracrine loop may exist for PTHrP/PTH1R in these tumours. The detection of PTHrP in the nucleus of rat buffalo hepatoma cells (MCA RH 7777), implies that nuclear transport of PTHrP may also occur in these cells. A possible paracrine or autocrine action of PTHrP in hepatoma cells has previously been suggested by Li et al who demonstrated that Hep G2 cells could synthesize and secrete both immunoreactive and biologically active PTHrP, and express messenger RNA for PTH1R.[clv] They reported that neutralization of endogenous PTHrP secreted by these cells, by the addition to the cell media of antiserum to PTHrP, resulted in increased cell growth, implying that that PTHrP had growth regulatory effects in these cells, in this case inhibitory properties.

The possibility of an autocrine/paracrine pathway for PTHrP/PTHrP, and any possible growth regulatory effects in HCC cells warrants further investigation. Despite advances in surgical and non-surgical treatments for HCC, the prognosis remains extremely poor. If the PTHrP/PTH1R system is found to have a pathophysiological role in HCC tumour growth, agents that target this system may be developed, which may have a therapeutic role in the management of these tumours.

# Chapter 5 EXPRESSION OF PARTHYROID HORMONE-RELATED PEPTIDE AND THE PARATHYROID HORMONE TYPE 1 RECEPTOR IN NORMAL LIVER, CIRRHOTIC LIVER, PUTATIVE HEPATOCELLULAR CARCINOMA PRECURSOR NODULES, AND HEPATOCELLULAR CARCINOMA

## 5.1 Introduction

The liver has been reported in several immunohistochemical studies to produce PTHrP. Most studies have observed immunoreactivity for PTHrP or detectable mRNA for PTHrP in the foetal human or rat liver, but only minimal or absent expression in the adult liver.[clxx, clxxi, clxxii, clxxiii, x] In a study by Kramer et al,[cviii] immunostaining for PTHrP was observed in the adult human liver, but was most prominent in blood vessels. Funk et al observed a low level of expression of PTHrP mRNA and protein in the adult rat liver, which increased markedly in response to inflammatory stimuli (endotoxaemia), where it was found to activate the hepatic acute phase response.[clxxiv]

In contrast, in the adult rat, the liver has been identified as a site of PTH1R expression, with relative abundant expression of receptor RNA levels. [clxxv, clxxvi, liii] A large body of literature exists, predating the discovery of PTHrP, describing possible hepatic effects of PTH in the liver of the adult rat and dog, [clxxvii, clxxviii, clxxix, clxxx, clxxxi, clxxxii, clxxxiii, clxxxiv, clxxxv] implying PTH1R expression.

The majority of patients who develop HCC have underlying cirrhosis, which suggests that cirrhosis itself represents a preneoplastic condition. Arakawa et al first reported in 1986 that an early HCC evolved in an adenomatous hyperplastic nodule.[clxxxvi] Subsequently a large number of studies of surgically resected livers were performed in Japan and in the West, and the general consensus reached amongst pathologists was

that histological changes preceding malignant transformation usually occur within dysplastic nodules, i.e. the emergence of hepatocellular carcinoma is a stepwise process. These histological changes include an increase in cellularity, fatty and/or clear cell changes, an irregular thin-trabecular pattern with frequent acinus and pseudogland formations, cell invasion within the fibrous stroma and vessel walls in cirrhotic livers,[clxix] and a stepwise increase in abnormal arterial luminal areas (intratumoral arterioles) [clxxxvii, clxxxviii.] Between 40 and 50% of dysplastic nodules are believed to undergo malignant transformation,[clxxxix,cxc] and therefore many pathologists are beginning to regard dysplastic nodules as malignant.[cxci] The early HCC is usually well differentiated, and within it evolves less well differentiated HCC.

The observation that PTHrP is expressed in foetal liver, expressed at a low level or not at all in adult liver, and then 'switched on' in hepatocellular carcinoma suggests that in liver, as in a number of other tissues, PTHrP may play a role as a regulatory factor for hepatocyte growth and differentiation. The purpose of these experiments were: 1) to investigate by immunohistochemistry the expression of PTHrP and its corresponding PTH1R in human resection specimens from normal liver, cirrhotic liver (regenerating nodules), macroregenerative nodules, dysplastic nodules and hepatocellular carcinomas; 2) to evaluate proliferative activity in the tissues (assessed by Ki-67 antigen expression); and 3) to assess whether there is a relationship between PTHrP/PTH1R expression and proliferative activity.

## 5.2 Materials and Methods

### 5.2.a Patient Tissue Specimens

Thirty-two cases of surgically resected livers were selected from the pathology archives of the Royal Free Hospital from May 1997 to November 2002 (Table 10). These cases included 13 macroregenerative nodules (MRN), 13 dysplastic nodules (DN) and 20 hepatocellular carcinomas (HCC). Non-neoplastic livers displayed regenerative nodules in 7 cases, and normal liver in 10 cases. Twenty-two of the 32 patients had been transplanted for cirrhosis of the liver. The cases of normal liver were from 9 patients who had undergone liver resection for colorectal metastatic adenocarcinoma, one case was from a transplant donor which had not been used for technical reasons. Macroregenerative nodules, dysplastic nodules and hepatocellular carcinomas were classified on the basis of morphological characteristics as previously described. All of the patients had been normocalcaemic pre-operatively. All patients had given consent for the use of liver tissue for research and under ethical approval by the Royal Free Hospital ethical committee. All of the liver specimens had been received within one hour after removal, sliced and fixed in formalin for 24 hours. Protocol samples were paraffin processed and tissue sections (H & E and reticulin stains) were prepared using routine histological laboratory standard procedures. Sequential sections (5μm thick) were cut with a microtome from the formalin-fixed, paraffin-embedded tissues, mounted on APES-coated slides, and dried overnight at 60°C.

*Table 10 Patient population, histological details, and aetiology of liver disease in the surgical resection specimens studied.*

| Case Number | Age (years) | Sex | Aetiology of Liver Disease | Type of Tissue |
|---|---|---|---|---|
| 1 | 46 | F | CRC | Normal liver |
| 2 | 64 | M | CRC | Normal liver |
| 3 | 64 | F | CRC | Normal liver |
| 4 | 64 | F | CRC | Normal liver |
| 5 | 68 | M | CRC | Normal liver |
| 6 | 50 | M | CRC | Normal liver |
| 7 | 72 | M | CRC | Normal liver |
| 8 | 65 | F | CRC | Normal liver |
| 9 | 55 | F | CRC | Normal liver |
| 10 | 19 | M | CRC | Normal liver |
| 11 | 64 | M | HCV | RN |
| 12 | 63 | M | ALD | RN, HCC |
| 13 | 39 | M | HCV | RN, MRN, DN |
| 14 | 56 | M | ALD | RN, DN(x2), HCC(x2) |
| 15 | 48 | M | HBV/ALD | RN |
| 16 | 42 | M | HBV/HDV | RN |
| 17 | 58 | M | ALD | RN |
| 18 | 54 | M | HBV | MRN(x5) |
| 19 | 41 | M | HCV, AATD | MRN (x2), DN (x4), HCC (x4) |
| 20 | 60 | M | HCV | MRN |
| 21 | 55 | M | HBV, HCV | MRN |
| 22 | 58 | M | HBV | MRN, DN, HCC |
| 23 | 68 | M | ALD | MRN, HCC (x2) |
| 24 | 54 | M | ALD | MRN, DN |
| 25 | 61 | M | ALD,HCV | DN (x3), HCC (x3) |
| 26 | 46 | M | HCV | DN |
| 27 | 45 | F | WD | HCC |
| 28 | 62 | M | HBV | HCC (x2) |
| 29 | 61 | M | ALD | HCC |
| 30 | 53 | M | HCV | HCC |
| 31 | 53 | M | HCV | HCC |
| 32 | 60 | M | HBV | HCC |

**Legend:** F = female, M = male, CRC = colorectal metastatic adenocarcinoma, HBV = Hepatitis B virus, HCV = Hepatitis C virus, HDV = Hepatitis D virus, ALD = Alcoholic liver disease, AATD = alpha-1 antitrypsin deficiency, WD = Wilson's disease, RN = regenerative nodule, MRN = macroregenerative nodule, DN = dysplastic nodule, HCC = hepatocellular carcinoma.

### 5.2.b Immunohistochemistry

Sequential sections were tested with anti-PTHrP (1-10) (Aphton), anti-PTH1R (Lab Vision), and anti-Ki67 (DAKO) antibodies, at the same concentrations, and using the same methods and as outlined previously. After pretreatments, endogenous avidin-binding activity was blocked with an avidin-biotin block as described previously (Chapter 4.2.b.1). All experiments were performed at room temperature, and were validated by the inclusion of positive control sections. Negative control sections were made by substitution of the primary antibody with normal mouse serum; for the anti-PTHrP antibody, specificity of staining was further validated by the inclusion of a parallel section of tissue that was incubated with a primary antibody solution antibody pre-absorbed with immunizing peptide.

Evaluation of PTHrP/PTH1R staining

Sections were examined by light microscopy and scored by two independent observers using the previously described method (chapter 2.2.d, chapter 4.2.b.2).

Evaluation of Ki67 staining

A Zeiss Axioscope 2 microscope and an axiocam with axiocam software Axiovision 3.0 was used to photograph four 20x magnification fields from each lesion. Photographs were printed on A4 sheets. The total number of recognisable lesional tumour nuclei was counted in each photograph. Nuclei showing distinct immunostaining were counted as positive. The labelling index was calculated as the percentage of positive nuclei out of the total number of nuclei counted. For statistical analyses, percentages were grouped as being: 1 = <1%, 2 = 1-5%, 3 = 6-10%, 4 = 11-30%, 5 = 31-50%, 6 = >50%.

Statistical analyses were performed using the Spearman rank correlation test, and the Mann-Whitney U non-parametric test for group differences as described in Chapter 2.2.e.

## 5.3 Results

Immunostaining for PTHrP and PTH1R was evident only in the cytoplasm of liver cells, tumour cells and bile ductules. Pre-absorption of the antibody with the immunizing peptide abolished all staining for PTHrP. All staining for PTH1R was abolished by substitution of normal mouse serum for primary antibody.

### 5.3.a Normal Liver

In all cases, hepatocytes showed diffuse weak staining for PTHrP with increased expression in the hepatocytes around central veins, and less expression in the hepatocytes around portal veins and tracts [median score 3 (range 2-4)] (Figure 20a). In the majority of cases (8 of 10) there was weak patchy staining for PTH1R in the hepatocytes, and no staining in 2 cases [median score 1 (range 0-1)] (Figure 20b). In the normal ducts there was strong staining for PTHrP [median score 6 (range 4-6)] and weak or absent staining for PTH1R [median score 1 (range 0-1)].

### 5.3.b Regenerating Nodules

In all the cases of regenerative nodules studied, there was diffuse weak or moderate staining for PTHrP [median score 4 (range 3-6)], and in 6 of 7 cases diffuse weak staining for PTH1R [median score 3 (range 3-4)] (Figures 21 a & b). In the ducts, there was predominately strong staining for PTHrP [median score 6 (range 4-9)]), and weak or moderate immunostaining for PTH1R [median score 3 (range 1-4)].

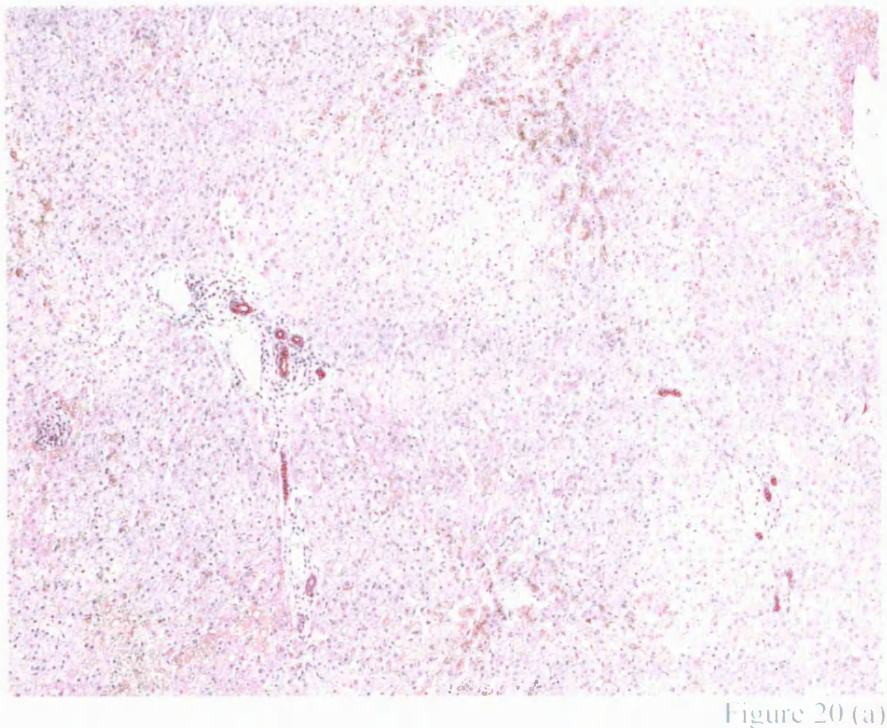


Figure 20 (a)

Figure 20 Immunohistochemistry showing weakly positive cytoplasmic staining for PTHrP (figure a) and PTH1R (figure b) in hepatocytes in a resection specimen of normal human liver.

There is strong staining for PTHrP in normal ducts. Photographed at magnification x50.

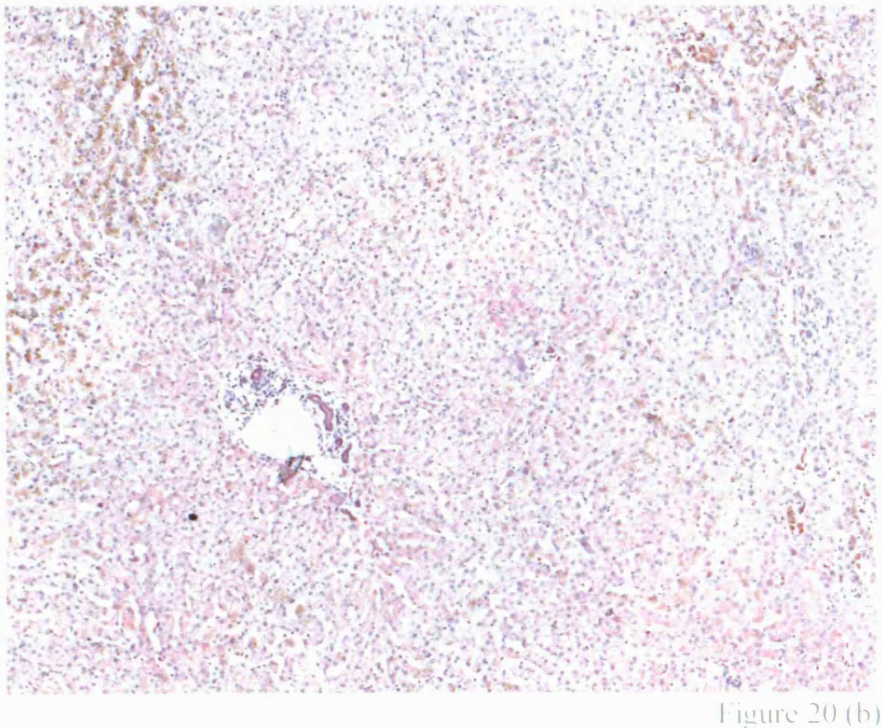


Figure 20 (b)

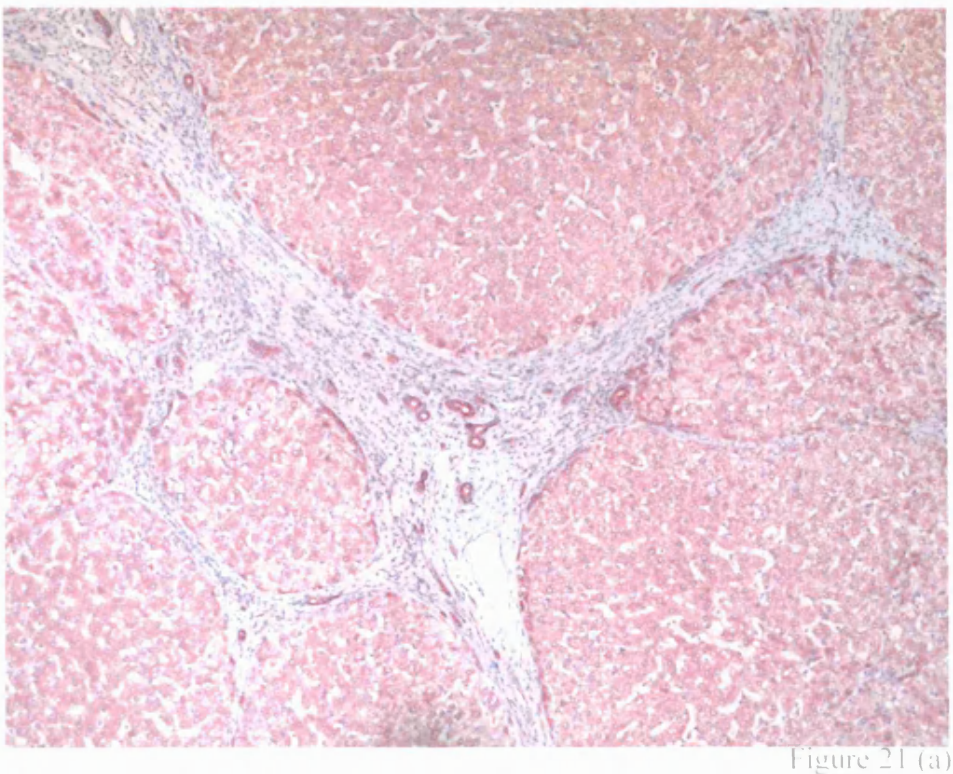


Figure 21 (a)

Figure 21 Immunohistochemistry showing positive cytoplasmic staining for PTHrP (figure a) and PTH1R (figure b) in regenerating nodules in a resection specimen of cirrhotic liver.

Normal ducts stain strongly for PTHrP and weakly for PTH1R. Photographed at magnification x50.

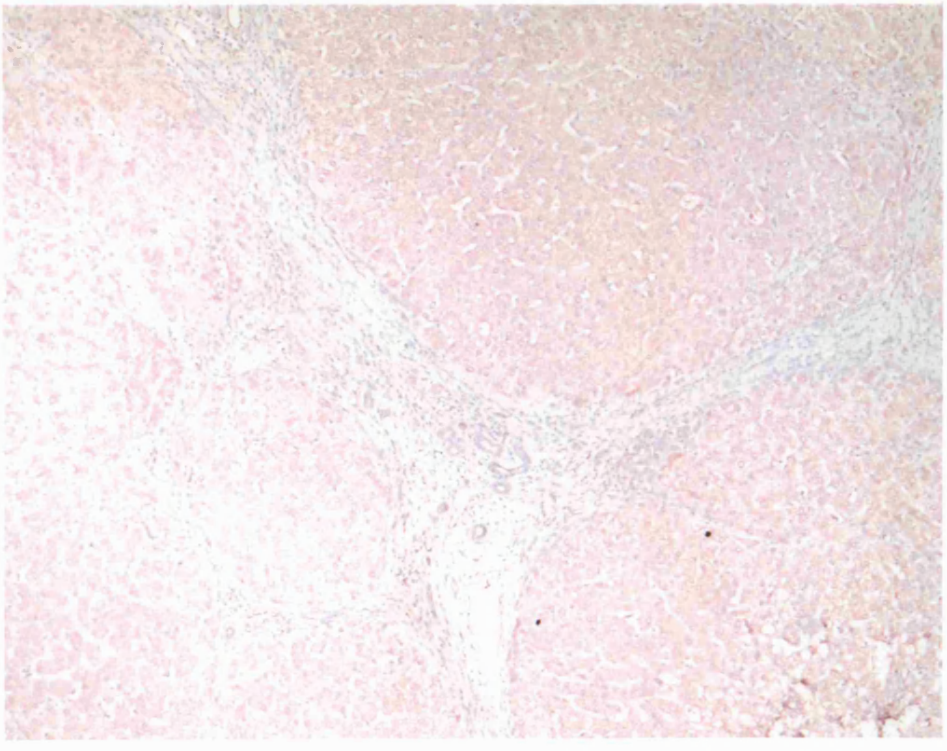


Figure 21 (b)

### 5.3.c Macroregenerative Nodules

In macroregenerative nodules, 5 of 13 cases displayed diffuse weak immunostaining for PTHrP, and the rest showed either diffuse moderate intensity staining or weak with areas of stronger staining [median score 4 (range 3-6)] (Figure 22a). In the majority of the cases (9 of 13), there was diffuse weak or moderate immunostaining for PTH1R in the nodules, and in the rest of the cases, weak patchy staining for PTH1R [median score 3 (range 1-4)] (Figure 22b). In the surrounding cirrhotic liver there was diffuse weak or moderate staining for PTHrP [median score 3 (range 3-6)] and diffusely weak or patchy immunostaining for PTH1R [median score 3 (range 1-4)]. In the ducts, there was predominately strong staining for PTHrP [median score 6 (range 3-6)]), and weak immunostaining for PTH1R [median score 1 (range 1-4)].

### 5.3.d Dysplastic Nodules

Six of the 13 cases of dysplastic nodules showed diffuse staining for PTHrP of moderate intensity, and 7 showed patchy moderate or weak staining [median score 4 (range 3-6)] (Figure 23a). In 5 cases the staining in the nodule was stronger than the staining in the surrounding cirrhotic tissue where hepatocytes showed diffusely weak or patchy moderate staining for PTHrP [median score 4 (range 3-6)]. The dysplastic nodules showed diffuse or patchy weak staining for PTH1R [median score 1 (range 1-4)] (Figure 23b). Background cirrhotic liver showed diffusely weak or patchy immunostaining for PTH1R [median score 3 (range 1-4)]. In the ducts, there was predominately strong staining for PTHrP [median score 6 (range 3-9)]), and weak or absent immunostaining for PTH1R [median score 1 (range 0-3)].

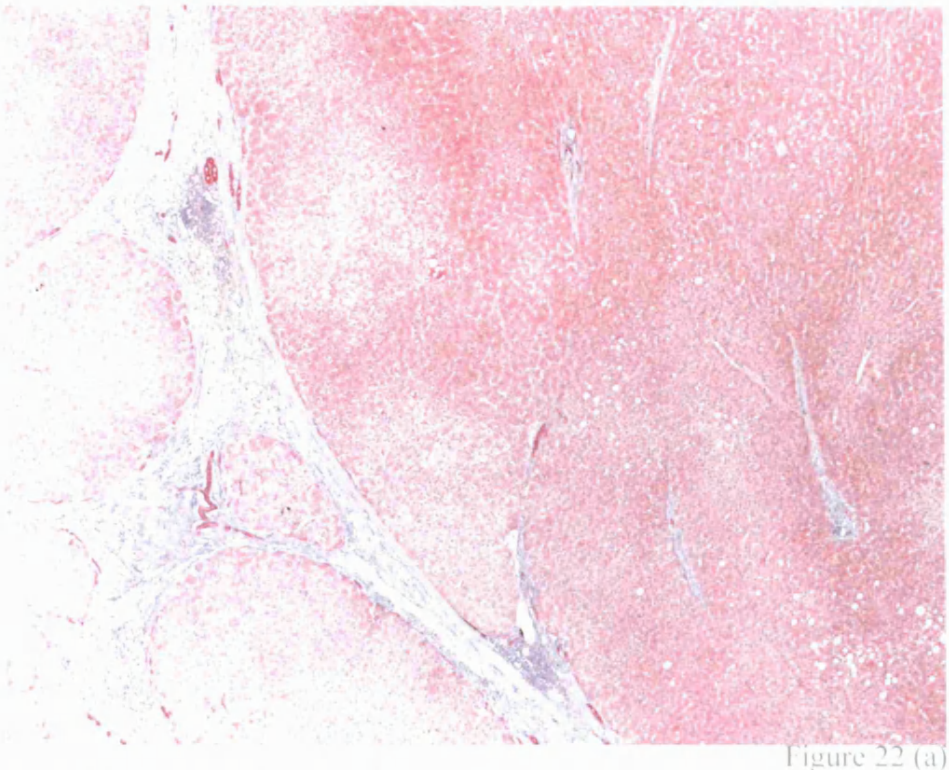


Figure 22 (a)

Figure 22 Immunohistochemistry showing positive cytoplasmic staining for PTHrP (figure a) and PTH1R (figure b) in a macroregenerative nodule within a resection specimen of cirrhotic liver containing a macroregenerative nodule.

In adjacent cirrhotic liver staining for PTHrP is weaker than in the macroregenerative nodule. Photographed at magnification x 25.

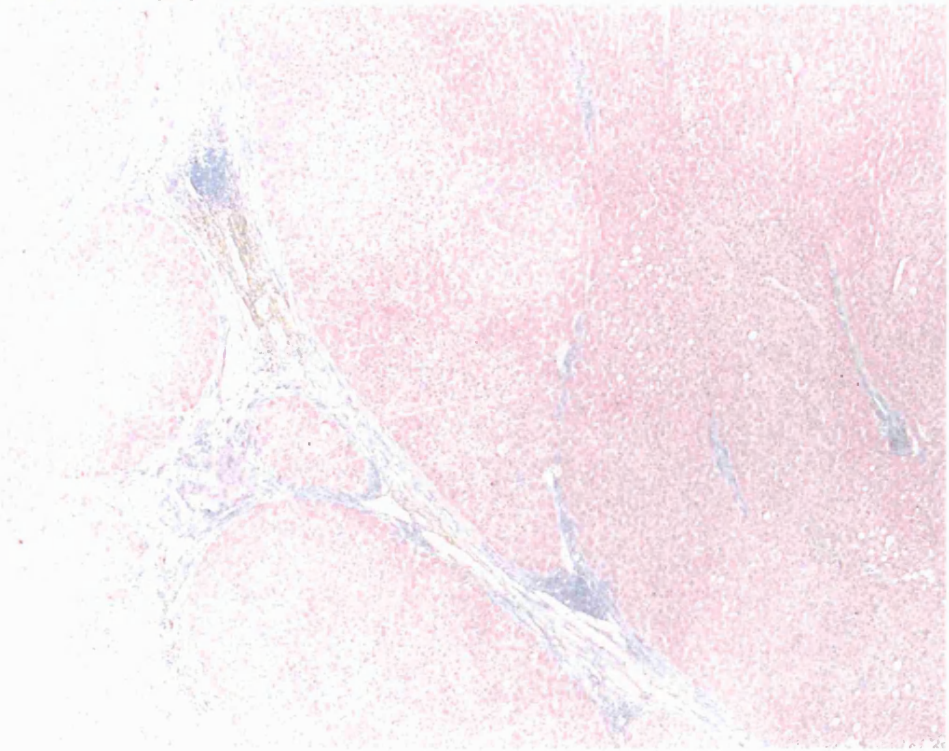


Figure 22 (b)

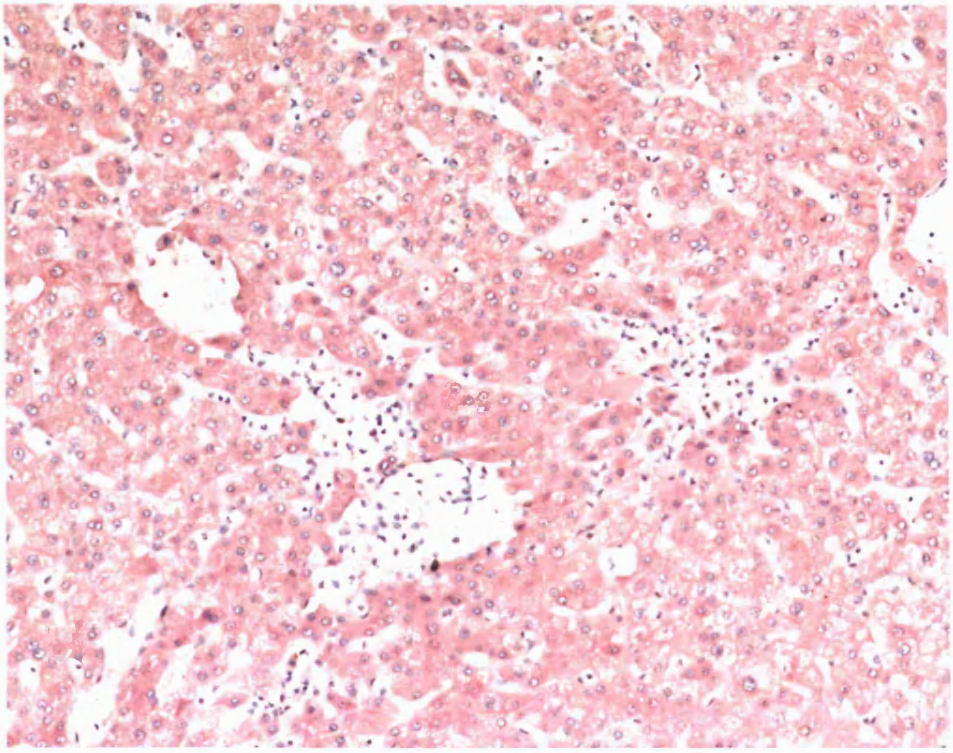


Figure 23 (a)

Figure 23 Immunohistochemistry showing positive cytoplasmic staining for PTHrP (figure a) and PTH1R (figure b) in a dysplastic nodule from a resection specimen. Photographed at magnification x100.

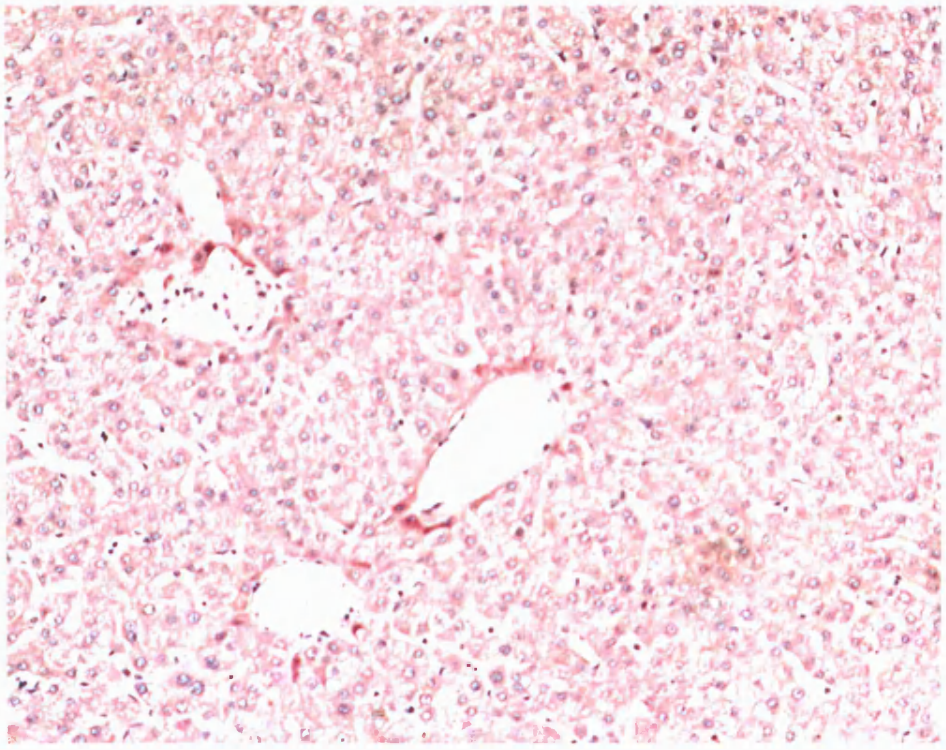


Figure 23 (b)

### 5.3.e Hepatocellular Carcinoma

All 20 cases of HCC showed diffusely positive staining for PTHrP of moderate intensity with areas of stronger intensity [median score 6 (range 4-6)], and staining for PTH1R that was diffusely weak or of patchy moderate intensity [median score 3 (range 1-6)] (Figures 24 a & b). In background liver, immunostaining for PTHrP was diffusely weak or moderate [median score 4 (range 1-6)] and staining for PTH1R was weak or in one case absent [median score 3 (range 0-3)]. In the ducts, again, there was predominately strong staining for PTHrP [median score 6 (range 3-9)]), and weak or absent immunostaining for PTH1R [median score 1 (range 0-4)].

Figure 24 Immunohistochemistry showing positive tumour cell staining for PTHrP (figure a) and PTH1R (figure b) in a resection specimen of hepatocellular carcinoma.

Photographed at magnification x 50.

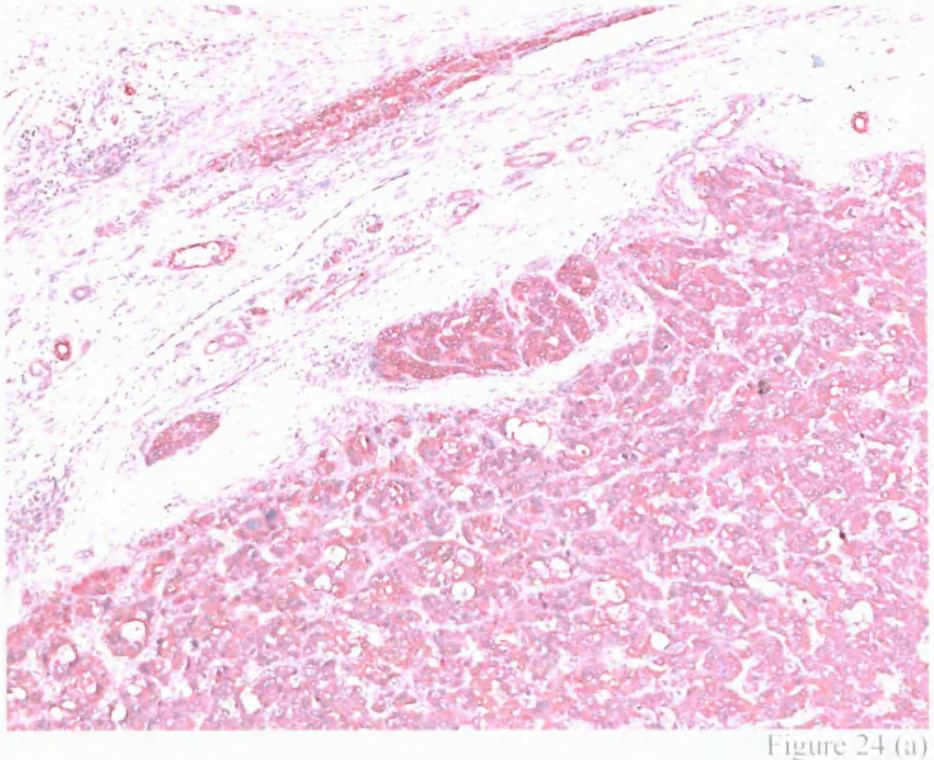


Figure 24 (a)

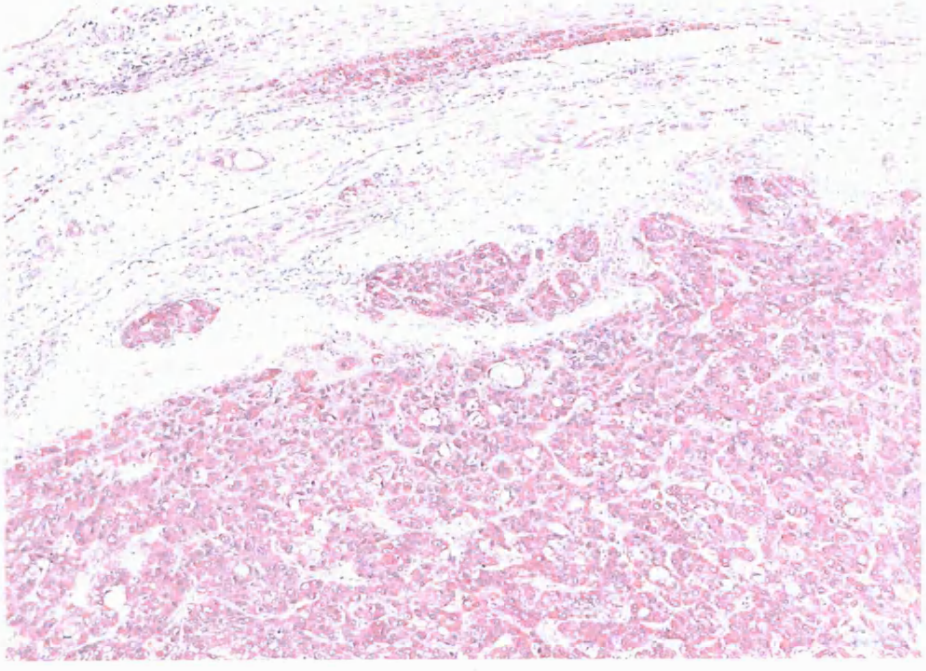


Figure 24 (b)

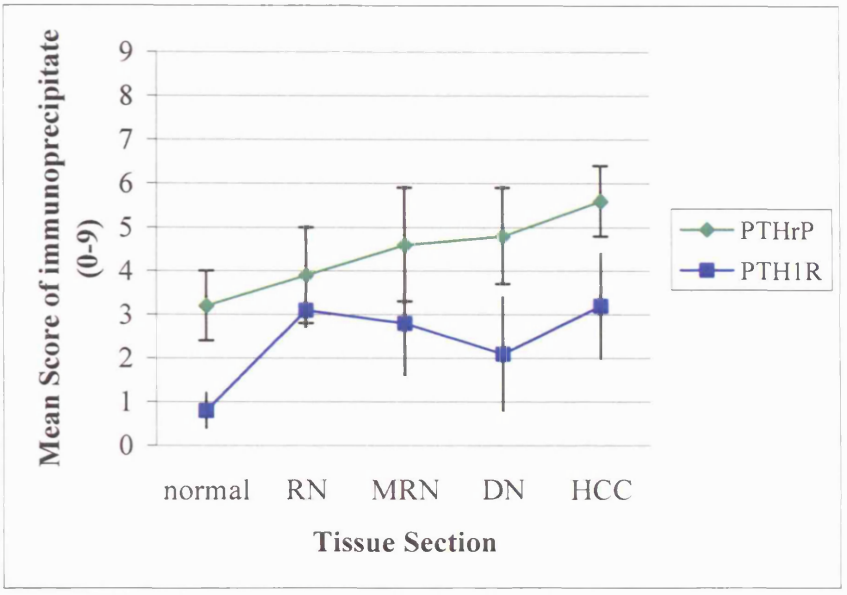
The mean immunostaining score for PTHrP was highest in the HCC tumour cells and then sequentially decreased through the dysplastic nodule group, to the macroregenerative nodules, to regenerative nodules to normal heptocytes (Graph 1). The intensity of staining for PTHrP was significantly greater in HCC tumour cells compared to the intensity of staining for PTHrP in dysplastic nodules ($p = 0.039$), macroregenerative nodules ($p = 0.007$), regenerative nodules ($p = 0.001$), and normal hepatocytes ($p < 0.001$), and significantly greater in dysplastic nodules and macroregenerative nodules compared to normal hepatocytes ($p = 0.002$ and $p = 0.032$ respectively). The difference in immunostaining scores for PTHrP between dysplastic nodules versus macroregenerative nodules and regenerative nodules, macroregenerative nodules versus regenerative nodules, and regenerative nodules versus normal hepatocytes, was not statistically significant. All of the different tissues showed immunopositivity for PTHrP of moderate intensity in the bile ductules. There was no significant difference in the ductular expression of PTHrP between the different tissues. The differences in mean intensity of staining for PTH1R across the tissue groups were not statistically significant. There was a significant positive correlation between PTHrP and PTH1R expression across the tissue groups ($r = 0.48$, $p < 0.001$).

*Table 11 Median and mean immunostaining product in non-lesional hepatocytes and ducts, and neoplastic cells in thesurgical resection specimen studied.*

| | | Non-lesional hepatocytes | | | Non-lesional bile ducts | | Macroregenerative nodule/dysplastic nodule/tumour | | |
|---|---|---|---|---|---|---|---|---|---|
| | | **PTHrP (0-9)** | **PTH1R (0-9)** | **Ki67 (%)** | **PTHrP (0-9)** | **PTH1R (0-9)** | **PTHrP (0-9)** | **PTH1R (0-9)** | **Ki67 (%)** |
| Normal liver (n =10) | Median (range) | 3 (2-4) | 1 (0-1) | 0.1 (0-1) | 6 (4-6) | 1 (0-1) | – | – | – |
| | Mean (SD) | 3.20 (±0.79) | 0.80 (±0.42) | 0.1 (±0.32) | 5.80 (±0.63) | 0.80 (±0.42) | – | – | – |
| RN (n = 7) | Median (range) | 4 (3-6) | 3 (3-4) | 0.8 (0-5) | 6 (4-9) | 4 (1-4) | – | – | – |
| | Mean (SD) | 3.86 (±1.07) | 3.14 (±0.38) | 1.54 (±1.90) | 6.14 (±1.46) | 2.71 (±1.25) | – | – | – |
| MRN (n =13) | Median (range) | 3 (3-6) | 3 (1-4) | NA | 6 (3-6) | 1 (1-4) | 4 (3-6) | 3 (1-4) | 0.98 (0-11.55) |
| | Mean (SD) | 4.08 (±1.38) | 2.54 (±1.20) | NA | 5.62 (±0.96) | 1.77 (±1.24) | 4.46 (±1.33) | 2.77 (±1.17) | 3.09 (±0.05) |
| DN (n =13) | Median (range) | 4 (3-6) | 3 (1-3) | NA | 6 (3-9) | 1 (0-3) | 4 (3-6) | 1 (1-4) | 3.62 (0-37.08) |
| | Mean (SD) | 4.31 (±1.03) | 2.15 (±0.99) | NA | 5.46 (±1.61) | 0.92 (±0.73) | 4.85 (±1.14) | 2.08 (±1.26) | 7.57 (±0.09) |
| HCC (n =20) | Median (range) | 4 (1-6) | 3 (0-4) | NA | 6 (3-9) | 1 (0-4) | 6 (4-6) | 3 (1-6) | 7.5 (1–40) |
| | Mean (SD) | 4.20 (±1.61) | 2.20 (±1.15) | NA | 5.75 (±1.58) | 1.35 (±1.31) | 5.60 (±0.82) | 3.15 (±1.18) | 11.99 (±0.11) |

**Legend:** RN = regenerative nodules, MRN = macroregenerative nodules, DN = dysplastic nodules, HCC = heptocellular carcinoma, SD = standard deviation, NA = not assessed

Graph 1 Mean intensity of staining for PTHrP and PTH1R in normal liver hepatocytes, regenerative nodules (RN), macroregenerative nodules (MRN), dysplastic nodules (DN), and hepatocellular carcinoma (HCC) tumour cells.



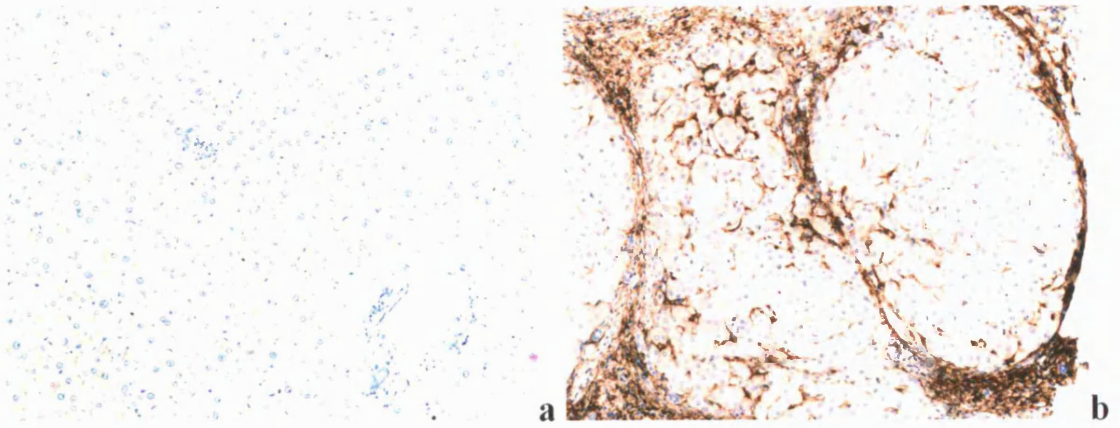

### 5.3.6 Expression of Ki67

A progressive increase in Ki67 from regenerative to macroregenerative to dysplastic nodules to HCC was observed (Figure 25, Table 11, Graph 2). Ki67 scores were significantly higher in dysplastic nodules compared to regenerative and macroregenerative nodules ($p = 0.03$ and $p = 0.04$ respectively), and also in HCC compared to regenerative and regenerative nodules ($p = 0.001$ and $p = 0.001$ respectively). There was a low level of expression of Ki67 (1%) in hepatocytes in only one case of normal liver.

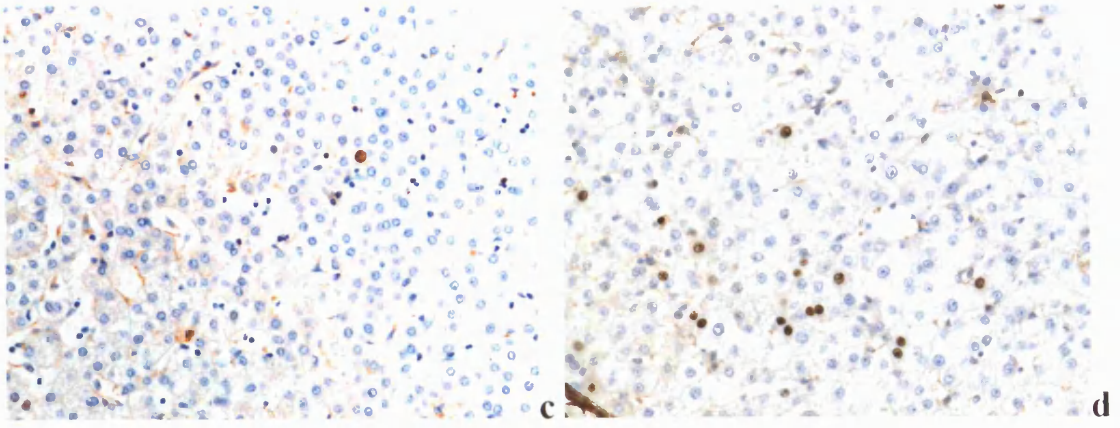
There was a positive correlation between the Ki67 score and the staining index for PTHrP ($R = 0.430$, $p = 0.001$, $n = 59$) and PTH1R ($R = 0.324$, $p = 0.012$, $n = 59$) across all tissue groups.

Figure 25 Immunohistochemistry for Ki67 in resection specimens of normal liver (a), regenerating nodules (b), macroregenerative nodule (c), dysplastic nodules (d), and hepatocellular carcinoma (e & f).

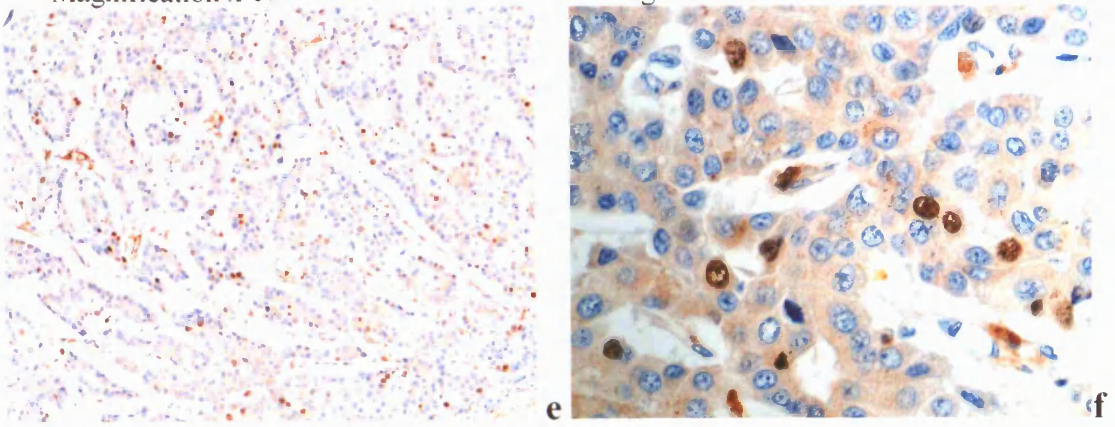
Positive staining is nuclear and appears as a brown precipitate.




Magnification x 50 Magnification x 50

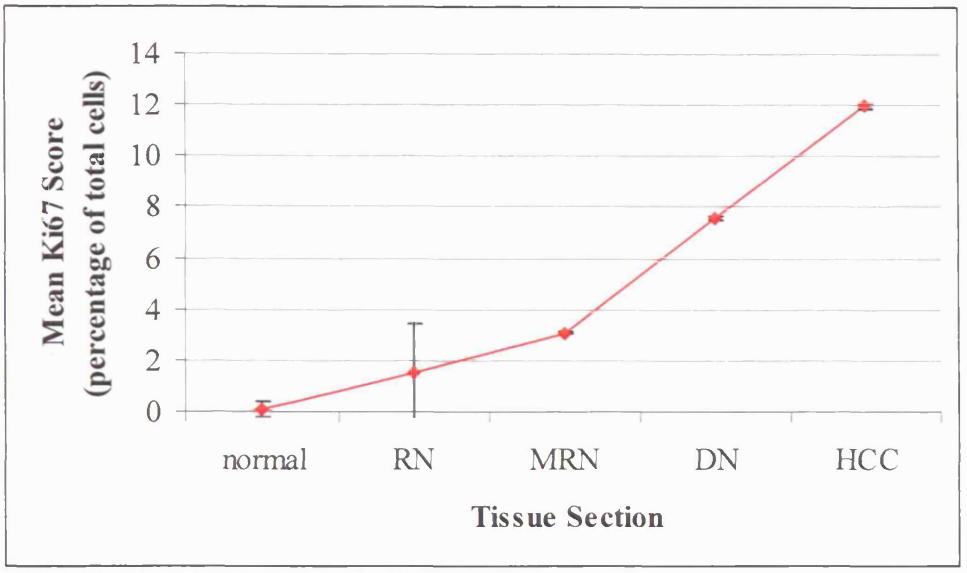



Magnification x 100 Magnification x 50




Magnification x50 Magnification x400

Graph 2 Mean Ki67 score in normal liver hepatocytes, regenerative nodules (RN), macroregenerative nodules (MRN), dysplastic nodules (DN), and hepatocellular carcinoma (HCC) tumour cells.




## 5.4 Conclusions

Immunopositivity for PTHrP was sequentially increased from normal liver, through cirrhotic liver and macroregenerative nodules, to dysplastic nodules, with a gradient of expression that peaked in malignant cells. This increased expression, supported by the positive correlation between PTHrP expression and expression of the proliferation marker Ki67, qualifies PTHrP as a likely candidate to influence hepatocyte and tumour cell growth. The positive correlation between PTHrP and PTH1R expression through the tissue groups supports the possibility of an autocrine/paracrine role for PTHrP/PTH1R in the development of regenerative, dysplastic nodules, and HCC.

Similarly, a gradient of increased PTHrP expression through normal to neoplastic and carcinomatous tissue has been observed in the prostate. PTHrP is expressed in normal prostatic tissue by neuroendocrine cells[i] and glandular epithelium. It is also expressed in benign prostatic hyperplasia (BPH) [cviii] and in epithelial cell cultures derived from these tissues,[ii] in the premalignant condition prostatic intraepithelial

neoplasia (PIN),[cxciv] and by most prostatic adenocarcinoma.[cxcv, cxcvi, cxcvii] Protein expression has been found to be at higher levels in prostate cancer than in BPH[cxcvii] or normal tissue,[cxcvi] and at significantly higher levels in PIN than in normal prostatic epithelium.[cxciv] PTH1R has been found by both immunohistochemistry and polymerase chain reaction to be robustly expressed in the majority of prostatic adenocarcinomas studied.[cxcviii] In prostate cancer cells PTHrP has been reported to play both autocrine/paracrine and intracrine roles, stimulating cell proliferation,[cxxxvii, cxxxviii] and protecting cells from apoptotic stimuli.[cxcix]

There was a low cellular proliferation rate as assessed by Ki67 expression in regenerative nodules, with a progressive increase to dysplastic nodules to HCC. This is consistent with previous studies showing increased proliferation rates in HCC and its precursor lesions.[cc, cci, ccii] The continuous proliferative status of hepatocytes in cirrhotic liver is considered to be a facilitating factor for the development of HCC. Expression of proliferation markers in cirrhotic liver has been previously described[cciii] and indicates liver cell regeneration occurring in the context of cirrhosis. This observation has been followed by studies investigating the possible use of proliferation markers to define cirrhotic patients at higher risk of developing HCC.[cciv, ccv] Borzio et al showed that the hepatocyte proliferation rate, assessed by expression of silver-stained nuclear organizer regions (AgNOR), was strongly predictive of hepatocellular carcinoma development in the cirrhotic liver. Donato et al, assessing liver cell proliferative status by immunostaining for proliferating cell nuclear antigen (PCNA) in compensated cirrhotic patients observed that the proliferative rate seemed to predict the development of HCC. The low proliferative rate observed in the macroregenerative nodules, which are widely regarded to be precursor lesions to HCC, has also been previously described,[cc, cci] suggesting that their premalignant potential is not related to hyperplasticity. There was scanty immunostaining for Ki-67 in one histologically normal liver sample. This may be because the material was taken from the neighbourhood of a colorectal metastasis, and although it consisted of morphologically normal liver, some reactive hepatocellular change remains probable.

The positive correlation between PTHrP expression and Ki67 indices in dysplastic nodules and HCCs implies that the increased PTHrP expression by dysplastic and tumour cells may stimulate cell growth. This finding contradicts the *in vitro*

observations of Li et al.[clv] They reported that the hepatoma cell line HepG2 secreted biologically active PTHrP, which when neutralised by the addition of antiserum to PTHrP, increased cell growth. This effect was inhibited by the addition of synthetic PTHrP (1-36), and suggested that endogenously secreted PTHrP acted in an autocrine/paracrine fashion in HepG2 cells to suppress cell growth. However, this human hepatoma cell line is highly differentiated and is well documented to have properties that are characteristic of normal hepatocytes,[ccvi, ccvii] and so their observations may represent the effects of endogenous PTHrP in normal liver.

A growth inhibitory effect of N-terminal PTHrP peptides, acting via the autocrine/paracrine pathway, mediated by PTH1R, has been seen in the breast cell line MCF-7, [ccviii] and in vascular smooth muscle cells.[lxxvii] In these examples of tumour and physiological cell systems, PTHrP has also been shown to have a stimulatory effect independent of PTH1R via the intracrine pathway. In a study by Massfelder et al in an aortic vascular cell line, when cells were treated with amino terminus-containing peptides cell proliferation was inhibited. By contrast, when the same peptides were introduced into the cell by PTHrP transfections that also contained the nuclear localisation sequence, cell growth was stimulated. Deletion of either of the two localizing signals, PTHrP 88-91 and 102-106, abrogated the effect. Thus the overall effect of PTHrP on cell proliferation is dependent upon the net effect of the 'opposing' inhibitory autocrine/paracrine effects of PTHrP versus the stimulatory intracrine effects of PTHrP.

Another study by Massfelder et al in 2001[ccix] showed that these paradoxical trophic actions of PTHrP in vascular smooth muscle cells are reversed under pathophysiological conditions. They studied the effects of PTHrP in renovascular smooth muscle cells from normotensive rats (wild type) and spontaneously hypertensive rats (SHR). They showed that PTHrP mRNA and protein was increased in SHR-derived cells, and that PTH1R transcripts were similar in both cell lines. When exogenous PTHrP (1-36) was added to the cells, proliferation of wild type cells was decreased, but cell proliferation of SHR cells was increased. Whereas transfection with antisense PTHrP (1-139) cDNA, which abolished PTHrP mRNA, decreased wild type but increased SHR cell proliferation, i.e. PTHrP via the intracrine pathway was stimulating wild type cell proliferation but inhibiting SHR cell

proliferation. Additional studies by the same group indicated that the preferential coupling of PTH1R to G-protein $G_i$ in the SHR cells was responsible for the proliferative effect of exogenous PTHrP in these cells. PTH1R-dependent stimulation of $G_i$ protein by PTH has previously been shown to decrease activation of adenylyl cyclase, and hence the production of cyclic AMP,[ccx] which is acknowledged as the cellular pathway accounting for the antiproliferative effect of PTHrP in vascular smooth muscle cells.[lxxvii]

It is therefore possible that endogenously secreted PTHrP exerts an antiproliferative effect in normal liver, probably via PTH1R. Whereas in regenerating, dysplastic and hepatocellular cells, the effect of endogenously secreted PTHrP is to stimulate cell proliferation via PTH1R, perhaps by preferentially coupling of the receptor to $G_i$. In this series the average intensity of PTH1R staining rose sharply between normal and cirrhotic liver, levelled off or decreased in macroregenerative and dysplastic nodules, and increased again in HCCs. The apparent reduction in expression of PTH1R in MRNs and DNs may reflect down-regulation of the receptor in response to increased levels of presumably endogenous PTHrP interacting with cell surface receptors.

Further *in vitro* work, studying the effects on cell proliferation of exogenously added amino-terminal PTHrP peptides, and the effect of transfection with PTHrP anti-sense constructs, are required to define the autocrine and intracrine effects of PTHrP on hepatocellular carcinoma cell lines. These studies may then be extended to *in vivo* models to further define the action of PTHrP as a possible regulatory factor for hepatocyte growth and differentiation in the normal liver through to HCC.

# Chapter 6 UPTAKE AND CELLULAR PROCESSING OF PARATHYROID HORMONE-RELATED PROTEIN

## 6.1 Introduction

In previous parts of this study, immunoreactive amino-terminal PTHrP was detected in the nucleus of some tumour cell lines, and in the tumour cell nucleus in some resection specimens of human pancreatic adenocarcinoma. PTH1R was also detected by immunohistochemistry in the nucleus of the same resection specimens, and was found to be localised to the nucleus in pancreatic adenocarcinoma cell line BxPC3. In the majority of cell lines PTH1R was localised to the cytoplasm or cell membrane only.

The possible mechanisms by which the secretory protein PTHrP may gain access to the cytoplasm after secretion have been previously discussed (Chapter 1.7.a), and may involve endocytosis-dependent pathways. Endocytosis of PTH1R, with subsequent PTHrP transport and localisation to the nucleus has been demonstrated in some studies,[lxii, lxiii] but others have not supported this concept.[lxiv] Although immunodetection of PTH1R provides information about expression, and gives an indication of localisation of PTH1R protein, it does not give any information about functionality of the receptor protein.

The purpose of these experiments was to examine cellular uptake of fluorescently labelled exogenous amino-terminal PTHrP (1-34) by the prostatic adenocarcinoma cell line PC3, and the hepatocellular carcinoma cell line HepG2. PTHrP (1-34) binds to PTH1R with high affinity, and does not contain a nuclear localisation sequence. Uptake of labelled PTHrP (1-34) by these PTH1R-expressing cell lines can determine whether these cells express PTH1R with a functional binding domain, and furthermore examines whether PTHrP (1-34) can gain access to the nucleus via a PTH1R endocytosis-dependant pathway.

## 6.2 Materials and Methods

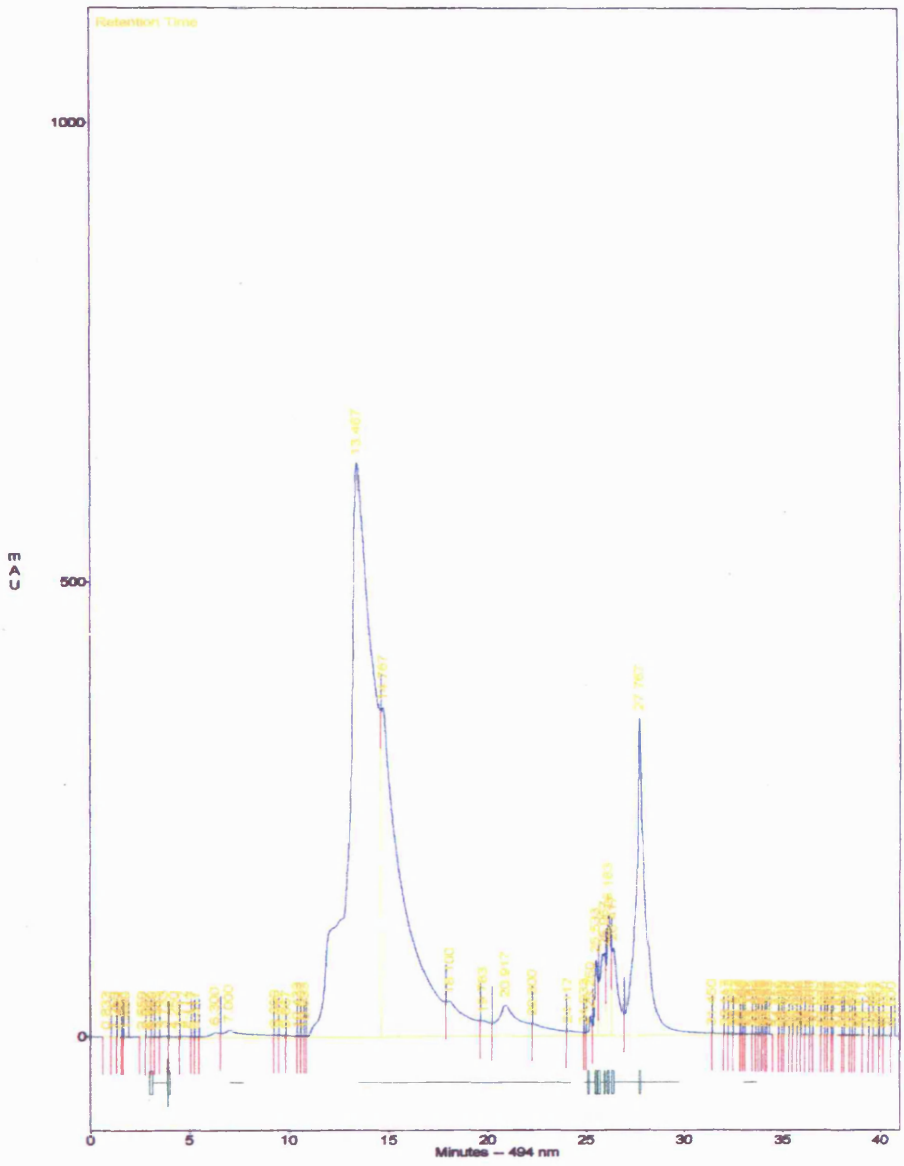
### 6.2.a Labelling of Peptide

An amino terminal PTHrP (1-34) peptide [Bachem (UK) Ltd., Merseyside, UK] was chosen for these experiments because it contains the amino acid sequences required for binding and activation of the PTH1R. This peptide was labelled with Alexa Fluor® 488 (Molecular Probes, Inc, Eugenes, OR, USA), a cadaverine label which is small, and water-soluble, with an absorption/emission maxima of ~ 495/519 nm, which can be fixed in cells by treatment with formaldehyde or glutaraldehyde.

### 6.2.b Removal of Free Dye

Preliminary uptake experiments were performed using labelled peptide that had been dialysed in sterile phosphate buffered saline (PBS) for 10 days at 4°C. However, in addition to cellular uptake, non-cellular background fluorescence was noted that was thought to due to the presence of free dye. For this reason, the labelled peptide was further analysed using reverse phase High Performance Liquid Chromatography (HPLC) [Beckman Coulter 168 Detector, Beckman Coulter (U.K.) Ltd] measured at 494nm. This showed 2 peaks: the first at 13 minutes, corresponding to the elution of free dye; and a second peak, corresponding to labelled peptide, with an elution time of 25-27.8 minutes (Figure 26).

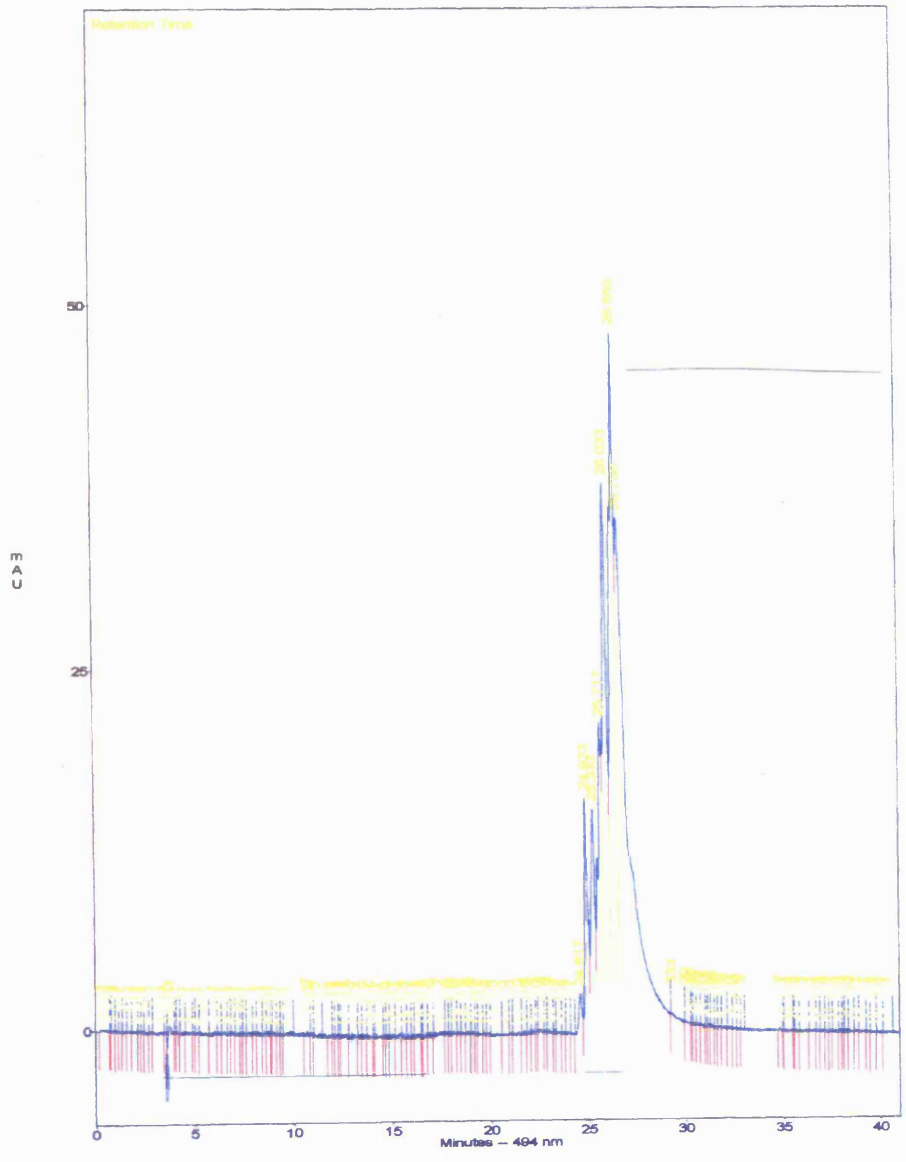
Figure 26 Analysis of PTHrP (1-34) labelled with Alexa Fluor® 488 by reverse phase High Performance Liquid Chromatography measured at 494 nm.
Two peaks are observed at 13 and 25-27.8 minutes.



Labelled peptide was therefore separated from the labelled peptide/free dye mixture by solid-phase extraction using a Sep-Pak C18 cartridge (Waters Associates, Milford, MA, USA). Briefly, prior to use, the Sep-Pak cartridge was rinsed with 5ml of aqueous methanol, followed by 5ml of distilled water. The peptide solution was passed through the cartridge, which was then flushed with distilled water until all free dye had been removed from the cartridge. The absorbed labelled peptide was then eluted from the cartridge with 1ml of 60% acetonitrile solution (Sigma-Aldrich Corporation, UK). The absorbance of the eluate was measured at 494nm. After purification only the later peaks, i.e. 25-27.8 minutes, were remaining. The peak at 13 minutes had disappeared, indicating that the free dye had been removed (Figure 27). Labelled peptide was separated from the 60% acetonitrate-water mixture used for elution by speed vacuum. The dry labelled peptide was stored at -20°C, and reconstituted with sterile PBS when required.

Figure 27 Analysis of PTHrP (1-34) labelled with Alexa Fluor® 488 by reverse phase High Performance Liquid Chromatography measured at 494 nm after solid-phase extraction of the labelled peptide/free dye mixture.

Peaks at 25-27.8 minutes are observed. The peak previously observed at 13 minutes has disappeared, indicating that free dye has been removed and only labelled peptide remains.



### 6.2.c Uptake Experiments

Uptake experiments were performed on PC3 cells and HepG2 cells. Cells were grown to 80% confluency in 8 chamber well plates in media containing 10% fetal calf serum (FCS). The cells were rinsed in PBS, and then Alexa Fluor labelled PTHrP (1-34), diluted in media to a final concentration of 0.02 mg/ml, was added to the cells to be incubated at 37°C for 5, 30 or 60 minutes. A negative control containing cells incubated in media alone was included in each experiment. The cells were then rinsed in PBS, and fixed in buffered formaldehyde for 5 minutes. Nuclei were counterstained with 3μg/ml 4',6 diamidino-2-phenylindole (DAPI) for 6 minutes, and mounted using Vectashield® mounting medium (Vector Laboratories, Ltd., Peterborough,UK ).The slides were examined using a Zeiss Axioscope fluorescence microscope with appropriate filters for Alexa Fluor 488 and DAPI fluorescence.

Uptake experiments were repeated with the same concentration of labelled PTHrP (1-34) in the presence of excess (0.2mg/ml) unlabelled PTHrP (1-34). This was done in order to determine whether the labelled peptide bound specifically to PTH1R, in which case its uptake would be reduced or attenuated in the presence of excess unlabelled peptide which competitively binds to the same receptor.

### 6.2.c Confocal Microscopy

Cellular uptake of labelled PTHrP (1-34) was further examined using confocal microscopy (FluoView™ 300 Confocal Microscope, Olympus). Optical sections were taken at 0.6-micrometre intervals perpendicular to the Z-axis (microscope optical axis) using a dual argon-ion (488 and 514 nanometer; blue/green) laser system.

## 6.3 Results

### 6.3.a Uptake Experiments of labelled PTHrP (1-34) by PC3 cells

After 5-minute incubation period with labelled peptide there was membranous uptake of labelled PTHrP (1-34). After 20-minute and 60-minute incubation periods, there was uptake of labelled PTHrP (1-34) to the cytoplasm and nucleus in up to 10% of cells (Figure 28). Uptake to these cellular structures was confirmed on confocal microscopy. Only minimal background cellular fluorescence was seen in the control well.

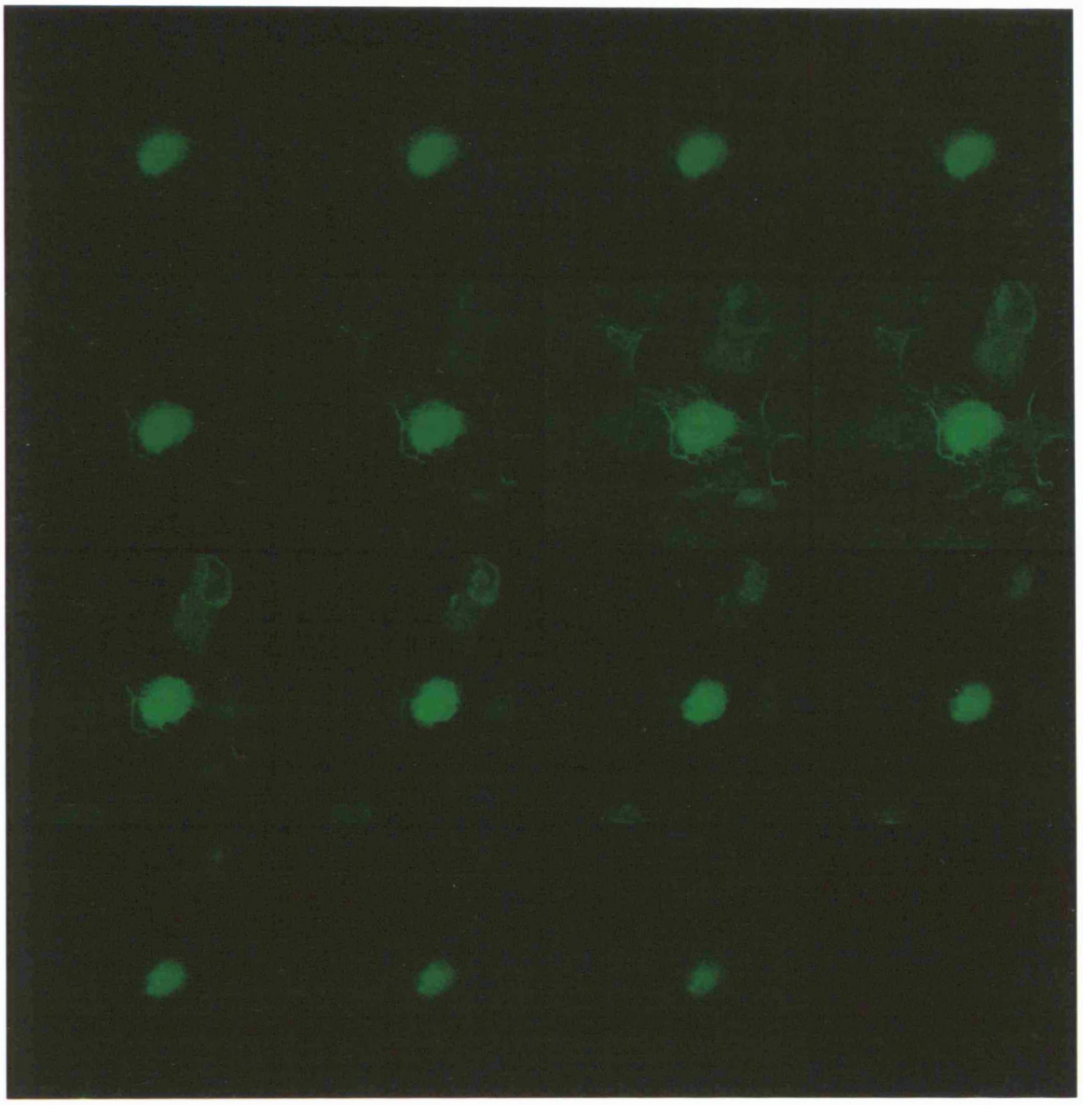


Figure 28 (a)

Figure 28 Series of confocal images representing interval sections of a PC3 cell after incubation with Alexa Fluor® labelled PTHrP (1-34) for 60 minutes.

The images in figure (a) show positive membranous, cytoplasmic and nuclear uptake of labelled PTHrP (1-34). Although only 12 of the 48 images collected through this series are presented in the figure, they represent individual focal planes separated by a distance of approximately 0.6 µm and provide a good indication of the internal cell structure.

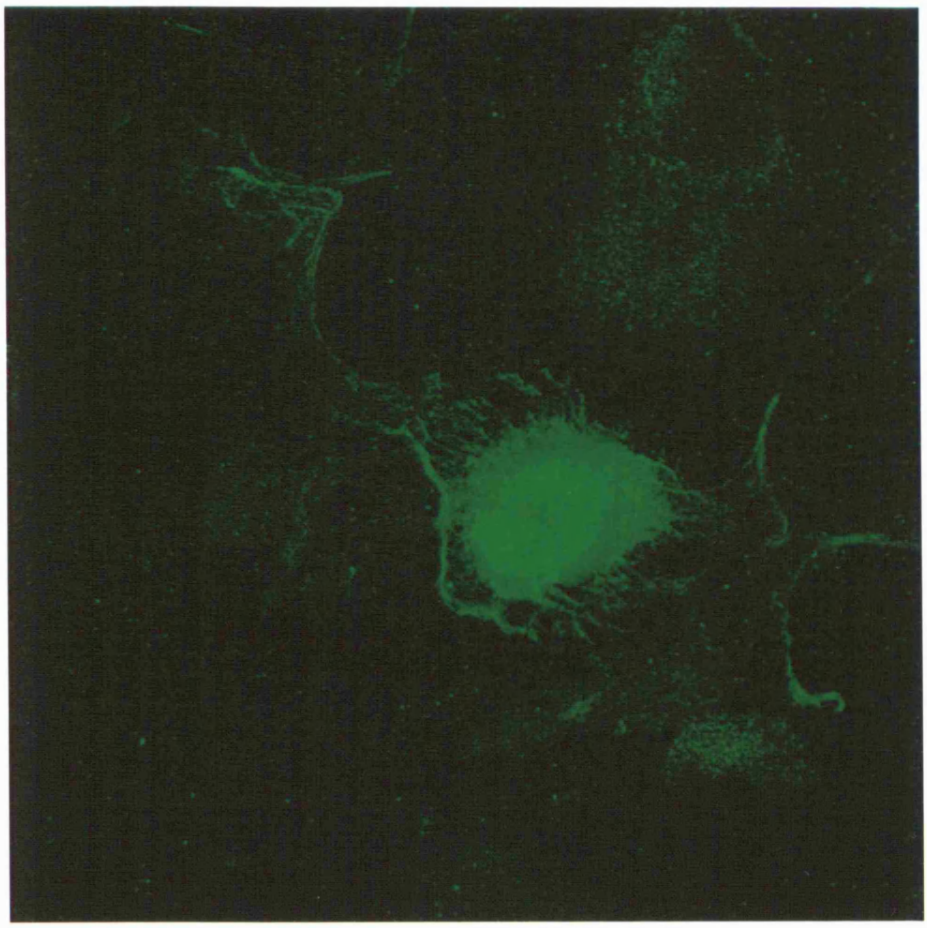


Figure 28 (b)

The confocal image in figure 28 (b) shows membranous and nuclear uptake of labelled PTHrP (1-34) in a PC3 cell.

In the presence of excess unlabelled PTHrP(1-34), there was reduced binding of labelled PTHrP(1-34) to the membrane, and no uptake to the cytoplasm or nucleus (Figure 29).

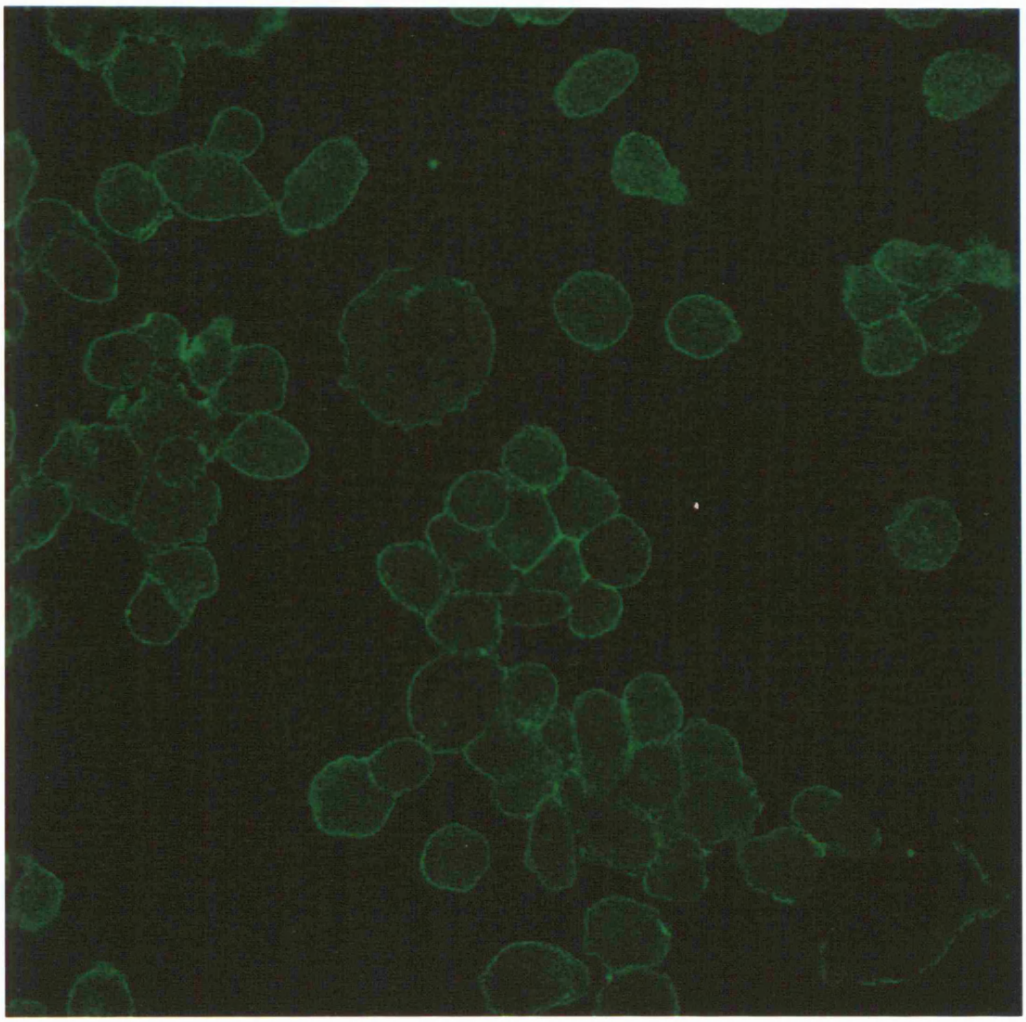


Figure 29

Figure 29 Confocal image of PC3 cells after incubation with Alexa Fluor labelled PTHrP (1-34) in the presence of excess unlabelled PTHrP (1-34).

After 20 minutes only membranous uptake of labelled PTHrP (1-34) is seen, with no uptake to the cytoplasm or nucleus.

### 6.3.b Uptake of labelled PTHrP (1-34) by HepG2 cells

Membranous uptake of labelled PTHrP (1-34) was observed after 5- and 20-minute incubation periods with labelled peptide. After 60-minute incubation with labelled peptide, membranous, cytoplasmic and perinuclear uptake of labelled PTHrP (1-34) was seen in up to 5% of cells (Figure 30). Uptake to these cellular structures was confirmed on confocal microscopy. Minimal background cellular fluorescence was observed in the control well.

Uptake experiments performed in the presence of excess unlabelled PTHrP (1-34), reduced binding of labelled PTHrP (1-34) to the membrane, and abolished cytoplasmic and perinuclear uptake.

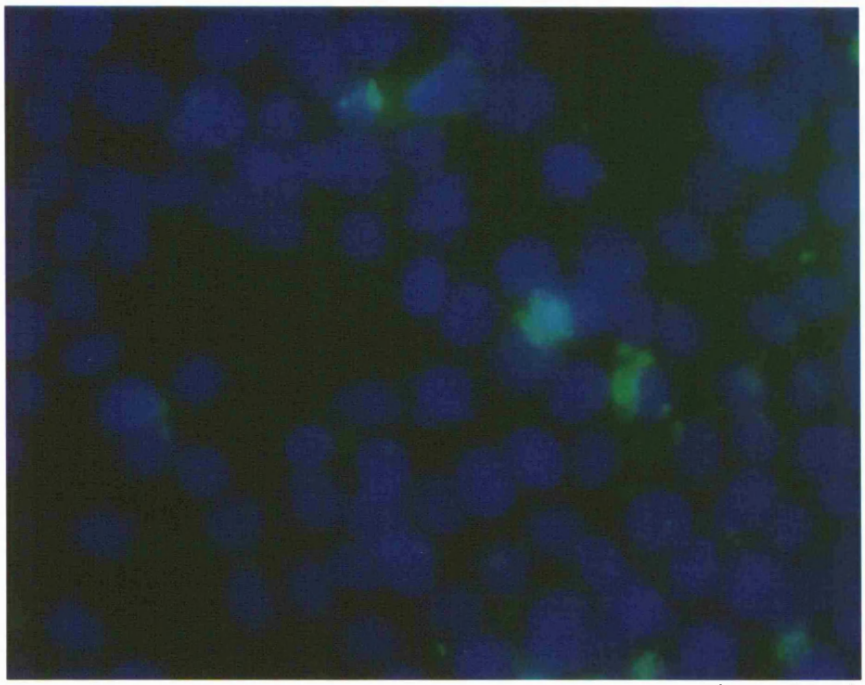


Figure 30 (a)

Figure 30 Images taken with an Axioscope fluorescence microscope represent uptake experiments with Alexa Fluor labelled PTHrP (1-34) on the hepatocellular carcinoma cell line HepG2.

Bound Alex Fluor 488 labelled PTHrP (1-34) fluoresce green, and the nuclei labelled with DAPI fluoresce blue. Images taken from the same representative field have been merged. Figure (a) shows membranous uptake of labelled peptide after incubation with labelled PTHrP (1-34) for 5 minutes at 37°C. Figure (b) shows membranous, cytoplasmic, and perinuclear uptake of labelled peptide after a 60-minute incubation with labelled PTHrP (1-34). Images were captured at x100 magnification.

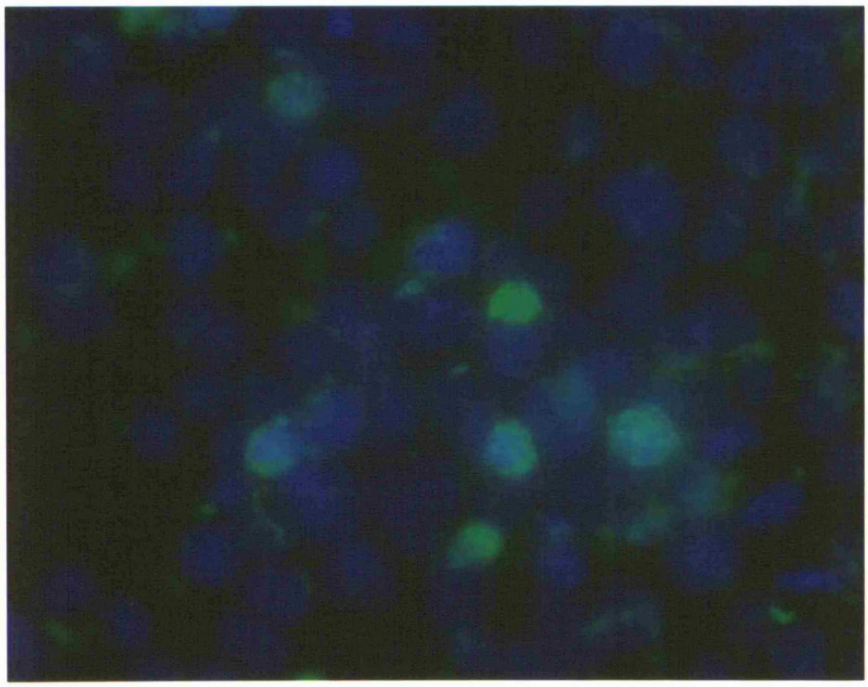


Figure 30 (b)

## 6.4 Conclusions

In the prostatic adenocarcinoma cell line PC3, fluorescently labelled PTHrP (1-34) bound to the cell membrane, was internalised, and subsequently transported to the nucleus. Internalisation of PTHrP (1-34) occurred through PTH1R-mediated endocytosis, as the presence of excess unlabelled PTHrP (1-34) competed with labelled PTHrP (1-34) for occupation and internalisation of available PTH1R, so attenuating uptake of labelled PTHrP (1-34). This demonstrated functionality of PTH1R in these cells, and suggests that PTH1R mediates not only internalisation of PTHrP, but also localisation to the nucleus.

Immunoreactive PTH1R has been demonstrated within the nucleus of various tissues in a broad range of cell types.[liii] Watson et al identified a nuclear localization sequence (NLS) between residues 446 and 473. By transfecting LLC-PK1 porcine kidney cells, which do not normally express PTH1R, with a vector containing the human wild-type PTH1R gene, they demonstrated that the transgene product entered the nucleus. When they transfected a vector containing a gene encoding a PTH1R without the NLS, however, the transgene product could only collect in a perinuclear ring. Thus, it seems that PTH1R has the ability to go directly to the nucleus, and that PTHrP might act on nuclear function through binding to intranuclear PTH1R.

The membranous binding of labelled PTHrP (1-34) to HepG2 cells, with subsequent cytoplasmic and perinuclear uptake after a 60-minute interval provides evidence that the PTH1R receptors expressed by these cells are also functional, and that PTH1R probably mediates PTHrP-endocytosis, but not translocation of PTHrP to the nucleus. Indeed, amino-terminal PTHrP was not detected immunohistochemically in the nucleus in this cell line (chapter 4.3.a.2). It is possible that the PTH1R expressed by HepG2 does not contain an NLS, and therefore cannot access the nucleus.

# Chapter 7 CONCLUSIONS AND CLINICAL IMPLICATIONS

These studies utilising immunohistochemistry demonstrate that both PTHrP and PTH1R protein are commonly expressed by tumour cells in human adenocarcinoma of the pancreas, neuroendocrine tumours of the gastroenteropancreatic axis, and hepatocellular carcinoma (HCC). PTHrP and PTH1R protein expression was further confirmed by western immunoblotting on cell lysates from different cell lines derived from these cancers. PTHrP and PTH1R expression has been documented in a number of human tumours, including breast, prostate, colon, and lung cancer. A paracrine/autocrine pathway for PTHrP/PTH1R has been reported to exist in these tumour systems in which PTHrP/PTH1R has been shown to have a role in neoplastic growth and development. The common expression of PTHrP and PTH1R in human pancreatic adenocarcinoma, gastroenteropancreatic neuroendocrine tumours, and HCC implies that an autocrine/paracrine pathway for PTHrP/PTH1R may exist in these tumours, which may also be involved in the regulation of cell growth and development.

If PTHrP does influence tumour cell growth, a relationship between the expression of PTHrP and cellular proliferation may be observed. This possible relationship was studied in hepatocellular carcinoma in chapter 5 using immunohistochemistry in resection specimens of human HCC. Cellular proliferation was examined by immunostaining for the nuclear proliferation antigen Ki67, and calculating the percentage of positive nuclei. PTHrP was measured by a semiquantitative method, which scored the intensity of the immunoproduct obtained for PTHrP, and the proportion of tumour cells with positive staining. A positive correlation between PTHrP staining and Ki67 staining was found ($R = 0.49$, $p = 0.028$), implying that PTHrP protein expression may influence, or be influenced by, cellular proliferation.

The majority of patients worldwide with HCC have underlying cirrhosis, from any cause, which predisposes to HCC.[ccxi] Hence cirrhosis can be considered to be a

premalignant condition. Patients who develop HCC almost always have chronic hepatitis, indicating that a chronic necroinflammatory condition is a key element of HCC occurrence. The presence of macroregenerative nodules has been evaluated as a morphological predictor of HCC in cirrhosis, and this high risk of developing HCC is further increased in the presence of high-grade dysplastic nodules. [ccxii] Up to 50% of dysplastic nodules are believed to undergo malignant transformation, and are therefore considered as precursor lesions to HCC.

In chapter 5, the expression of PTHrP and PTH1R was further examined in a series of resection specimens from normal liver, cirrhotic liver, macroregenerative nodules, dysplastic nodules, and hepatocellular carcinoma. Immunostaining was scored by the previously described method. A sequential increase in immunostaining for amino-terminal PTHrP was observed, from a low level of expression in normal liver, through cirrhotic liver and macroregenerative nodules, to dysplastic nodules, with a gradient of expression that peaked in malignant cells. This correlated with immunostaining for PTH1R across the tissue groups, supporting the existence of an autocrine/paracrine pathway for PTHrP/PTH1R in these tissues. The observation that PTHrP is expressed in foetal liver, expressed at low levels in adult liver, and then at higher levels ('switched on') in hepatocellular carcinoma, suggests that in liver, as in a number of other tissues, PTHrP may play a role as a regulatory factor for hepatocyte growth and differentiation. Immunostaining for the nuclear proliferation antigen Ki67 was also examined in these tissues. In regenerative and macroregenerative nodules there was low level of expression of Ki67, which increased in dysplastic nodules, with highest levels of expression in HCC tumour cells. Across the tissue groups, the gradient of PTHrP expression correlated Ki67 staining, suggesting that PTHrP, probably acting via PTH1R, is a likely candidate to influence hepatocyte and tumour cell growth.

Estimates from the year 2000 indicate that liver cancer (hepatocellular carcinoma and intrahepatic bile duct carcinoma) remains the fifth most common malignancy in men and the eighth in women worldwide.[ccxiii] There is considerable geographical variability in the incidence of primary liver cancer, largely explained by the distribution and natural history of the hepatitis B and C viruses. However during the last two decades, increases of primary liver cancer incidence rates have been reported from the United Kingdom,[ccxiv] Central Europe,[ccxv] North America, [ccxiii] Australia, and

Japan,[clxvi] accompanied by increases in mortality. The predominant cohort component of these increases have been interpreted as a long-term consequence of increased hepatitis C virus (HCV) exposures in the period 1960-1970 through contact with contaminated blood and intravenous drug use: the passage of 2 to 4 decades representing the 'incubation period' between HCV infection, and the development of cirrhosis and HCC. From this data it is predicted that the number of cases of HCC generated by the pool of HCV carriers will continue to increase for some time.

HCC remains a highly fatal cancer. The only curative therapies are surgical resection and transplantation. Both of these approaches can be successful, but recurrence of HCC is common, and unfortunately the vast majority of patients are not candidates for either treatment. This is often because patients with HCC present late in the course of the disease when the tumour is advanced and unresectable, or because they have underlying cirrhosis with inadequate hepatic reserve to tolerate extensive resection or cytotoxic therapy. Liver transplantation has been successful in treating limited-stage HCC, affecting cure on both the tumour and the underlying cirrhosis. However only a minority of patients qualify for transplantation, and with the present low availability of cadaveric donors, waiting times for transplant are long, leading to inevitable disease progression. Because patients at high risk of developing hepatocellular carcinoma, i.e. cirrhotic patients from any cause, and patients with chronic hepatitis B infection, can be identified, detection of HCC at an early stage by regular surveillance is possible and desirable. At present, monitoring of alpha-fetoprotein (AFP) and regular ultrasound scanning of the liver at six monthly intervals is recommended for cancer surveillance in patients with cirrhosis.[ccxvi] AFP has been used as a serum marker for HCC for many years, and has a sensitivity of 39-65%, specificity of 76-94%, and a positive predictive value of 9%-50%.[ccxvii] The poor sensitivity of AFP, particularly in the detection of small HCCs, is partly because elevated AFP levels may be seen in patients with cirrhosis or chronic hepatitis without HCC. Ultrasonography is operator dependent and limited in its ability to differentiate HCC from non-neoplastic lesions, such as macroregenerative nodules. Combined, AFP and ultrasound in general appears to be a more sensitive screening method, however it is not known with certainty whether screening with this method is able to reduce all cause mortality or HCC mortality. Clearly there is a need for novel strategies for the early detection of HCC in high risk patients.

In chapters 4 and 5, PTHrP protein was found to be expressed by HCC tumour cells in all the resection specimens and cell lines tested. There was no significant difference in immunostaining obtained for PTHrP between different tumour differentiation types, or tumour size (grouped for statistical evaluation into tumours that were < 20mm or ≥ 20mm), and whether or not there was histological evidence of vascular invasion, local invasion or multiple tumours present. PTHrP is undetectable in the circulation of normal men and women who are not pregnant, but if it is found to be elevated in the serum of patients with HCC, it may be a useful biomarker for HCC screening in high risk individuals. Most patients with HCC do not have hypercalcaemia, although hypercalcaemia can be expected in up to 10% of patients. Therefore it is likely that if PTHrP is detectable in the circulation of patients with HCC, it is present at lower levels than observed in patients with humoral hypercalcaemia. Because of the variety of molecular forms of PTHrP, linked to its catabolism, its immunoassay is difficult. However a number of immunoradiometric assays (IRMA) and radioimmunoassays (RIA), recognising the 1-72 and 1-86 domains respectively, are widely used, and have been validated in human subjects.[ccxviii, ccxix] If PTHrP is in low abundance in the circulation in patients with HCC, the detection limit of the assay used would accordingly have to be low. One method available to bypass the difficulty of detecting low levels of circulating proteins is the technique of immunodepletion, where the most abundant proteins in the serum, such as albumin and immunoglobulins, are removed from the serum.[ccxx] However, this technique would only be reliable if PTHrP was not bound to one of these major circulating proteins.

If PTHrP is released by HCC tumour cells into the extracellular compartment, and is present in detectable levels in the circulation of patients with HCC, in order to be a useful biomarker for screening it must be useful in distinguishing between HCC, and patients with cirrhosis or normal liver (Phase 1 study). Therefore serum levels of PTHrP in these patients would need to be studied. If significant differences between HCC and controls, i.e. patients with cirrhotic or normal liver, are found, a further comparison should be made to patients with early HCC [T1 (solitary, ≤ 2 cm, without vascular invasion) or T2 (solitary, ≤ 2 cm, with vascular invasion; multiple, one lobe, ≤ 2 cm, without vascular invasion; solitary, > 2 cm, without vascular invasion), in order to determine its usefulness as a biomarker that can detect early stage cancer.

Further determinations of the diagnostic capability of PTHrP as a biomarker for early stage HCC can then be made in phase 2 studies, which should include enough patients to adequately power the study, include controls which are patients for whom surveillance will be ultimately applied, i.e. patients with compensated cirrhosis without known HCC, and compare the results with currently available screening methods (AFP and ultrasonography). They should also estimate true-positive and false-positive rates for the clinical assay, and correlate results with covariants such as the cause of liver disease, and demographics. If phase 1 or 2 studies show promising results, a retrospective longitudinal study to detect preclinical disease (phase 3), prospective screening studies (phase 4), and cancer control studies (phase 5) may follow.

As a normal healthy cell transforms into a neoplastic cell, normal cellular protein expression is altered, and as a consequence, the flow of information by the proteome is changed. Altered protein expression is the effect of transcriptional and post transcriptional changes, post translational and structural modifications, and changes in the subcellular localisation of proteins. A large number of the proteins which are dysregulated in HCC undergo post translational alteration. In an analysis of 83 proteins identified by means of 2-dimensional polyacrylamide gel electrophoresis (2-D PAGE)[1] and mass spectroscopy, in HCC tissue, only 30% of protein changes correlated with changes at the corresponding transcript level, and notably 33% of proteins identified as modified in HCC corresponded to posttranslational protein cleavages.[ccxxi] Some of the proteolytic fragments generated were released into the extracellular compartment, and were detected in the sera of patients with HCC. Protein-processing dysregulation of PTHrP may occur which is specific to HCC. This may generate specific isoforms of PTHrP, and cleavage products in particular. This possibility could be further evaluated by analysis of HCC tissue using 2-D PAGE, followed by protein identification using mass spectroscopy. This may identify PTHrP species that could act as novel biological markers discriminating between HCC and other liver diseases that express PTHrP at lower levels, i.e. cirrhotic liver, macroregenerative and dysplastic nodules. However to be clinically useful, the candidate marker would have to be detectable in biological fluids such as plasma,

[1] This separates proteins first according to their isoelectric point, and then submits them to a second separation in relation to their mass.

serum and urine. If HCC does predominately generate and release into the circulation PTHrP isoforms that cannot activate PTH1R, or cleavage products of PTHrP that do not contain the amino-terminus, this may explain why humoral hypercalcaemia is not frequently seen in patients with HCC.

Because the majority of patients with HCC are not candidates for curative resection or liver transplantation, and current chemotherapy agents are largely ineffective and too toxic for these patients who usually have underlying cirrhosis, most therapeutic techniques are local ablative therapies, the development of which has recently undergone great progress. These techniques include chemoembolisation,[ccxxii, ccxxiii] percutaneous injection of ethanol,[ccxxiv] radiofrequency ablation,[ccxxv] focused ultrasound ablation,[ccxxvi] and intrahepatic infusion of ytrium-90 microspheres.[ccxxvii] However, although ablative therapies may slow the progression of cancer and prolong survival, they are unlikely to lead to cures or long-term remission in disease. Therefore new non-cytotoxic agents are need for HCC. A number of phase II trails have recently been published, reporting a growth-inhibitory effect of vitamin D analogues, administered either orally or directly with lipiodol via the hepatic artery in patients with inoperable HCC.[ccxxviii, ccxxix] Dalhoff K et al reported the results of an uncontrolled study of oral seocalcitol, a vitamin D analogue, given to 56 patients with inoperable advanced HCC. The dose was titrated according to serum calcium levels, and treatment effect was evaluated by regular CT scans. Out of 33 patients evaluable for tumour response, two had complete response (CR), 12 stable disease and 19 progressive disease. The CRs appeared after 6 and 24 months of treatment, and lasted for 29 and at least 36 months (one patient was still in remission when the data censored). Seocalcitol was well tolerated; the most frequently reported toxicity being hypercalcaemia, with associated symptoms. Any survival benefit with or without tumour response is still to be determined with controlled trials.

The vitamin D nuclear receptor is expressed in hepatocytes[ccxxx] and, more abundantly, in HCC cells. A number of vitamin D analogues have been shown to induce differentiation and inhibit tumour growth in hepatocellular carcinoma animal models and in HCC cell lines.[ccxxxi] Vitamin D has been shown to down-regulate the expression of PTHrP mRNA and secreted protein levels via a transcriptional system in the human prostate adenocarcinoma cell line PC3.[ccxxxii] Vitamin D3 has also been

shown to down-regulate PTH1R gene expression in a cell specific manner.[ccxxxiii] The observed growth-inhibitory effects of vitamin D analogues in hepatocellular carcinoma may involve inhibition of PTHrP/PTH1R expression by this hormone. This possibility could be investigated further by *in vitro* studies of the effect of vitamin D analogues on PTHrP/PTH1R mRNA and protein expression in HCC cell lines. Vitamin D analogues may have a therapeutic role in the treatment of HCC, and maintaining adequate plasma levels of vitamin D may be beneficial in the prevention and control of these tumours.

Pancreatic cancer also has a very high mortality, with an overall five-year survival of less than five percent, and is the fifth leading cause of death from cancer in the western world. The incidence of pancreatic cancer has steadily increased over the past decades. The UK has an especially high incidence of 10-12 per 100,000 population per year, with around 7,000 deaths per year. The reason for this high mortality is that these cancers often produce little in the way of symptoms until they are advanced and unamenable to curative surgical resection. Adenocarcinomas of the pancreas also disseminate early, show aggressive local tumour progression, and are resistant to treatment with chemotherapy and radiation.

Identification of patients with early pancreatic cancer should increase five-year survival. Patients with idiopathic and alcoholic pancreatitis, and new-onset diabetes mellitus (less than two years, no family history, age greater than fifty years) have a low, but increased risk of having or developing pancreatic cancer. Patients with hereditary pancreatitis who are older than forty-five years and have a paternal pattern of inheritance (estimated cumulative incidence to age seventy is approximately seventy-five percent),[ccxxxiv] intraductal papillary mucinous tumour (IPMT),[ccxxxv] familial pancreatic cancer, and pancreatic masses, have a high risk of developing pancreatic cancer. Ideally these patients should be carefully evaluated for pancreatic cancer. Unfortunately however, screening strategies for the detection of pancreatic cancer have been hampered by the low sensitivity of the currently available tumour marker Ca 19-9 in small stage 1 tumours, and the comparative lack of sensitivity of available radiological imaging. At present the most sensitive imaging test is endoscopic ultrasound (EUS),[ccxxxvi] however its sensitivity for detection of early pancreatic cancer is unknown, and it is still not widely available in the UK. The

sensitivity of Ca19-9 is between 70-86%,[ccxxxvii, ccxxxviii] depending upon cut-off levels, with a specificity of 87%. However, biliary obstruction with cholangitis caused by a lesion other than cancer also causes high serum levels of Ca 19-9.[ccxxxix] Currently the best that can be offered to patients at high risk of developing adenocarcinoma of the pancreas is spiral computed tomography (CT), followed by EUS if the CT results are undiagnostic, and measurement of Ca19-9.

In chapter 2, PTHrP was found to be expressed by tumour cells in the majority of resection specimens of human pancreatic adenocarcinoma, and in all human pancreatic carcinoma cell lines tested. If PTHrP is released by tumour cells into the extracellular compartment of patients with adenocarcinoma of the pancreas, and is present in detectable levels in serum, plasma or urine, it may be a useful tumour marker in these patients, particularly if it is produced at detectable levels in early stage 1 tumours. Again, in order to be a useful biomarker for screening it must be useful in distinguishing between pancreatic cancer and patients with chronic pancreatitis, normal pancreas, and from patients with cholangitis and biliary obstruction from benign pathology. Bouvet et al developed an orthotopic mouse model of the PTHrP-producing human pancreatic cell line, BxPC3, which had been transduced with green fluorescence protein so that tumour growth and metastases could be easily visualised. Serum PTHrP was measured by immunoassay at 5 and 13 weeks after tumour implantation, and was found to correlate with tumour burden, indicating that PTHrP could serve as a tumour marker in an animal model of pancreatic cancer.[ccxl]

Alternatively, protein-processing dysregulation of PTHrP may occur which is specific to pancreatic adenocarcinoma, generating specific isoforms and cleavage products of PTHrP. This possibility may be further evaluated, initially by analysis of pancreatic adenocarcinoma tissue using proteonomic techniques, such as 2-D PAGE followed by mass spectroscopy, multidimensional protein identification technology,[2] surface-

[2] This technique combines enzymatic digestion of complex protein mixtures, separation of the generated peptides by liquid chromatography, and then further analysis of the peptides by mass spectroscopy analysis. An advantage of this technique is the detection of low abundance protein, however a major limitation is the loss of information of potential modifications of the original protein.

enhanced laser desorption ionization (SELDI),[3] and protein microarray.[4, ccxxi] This may isolate PTHrP species that may become new biological markers that could discriminate between pancreatic adenocarcinoma and other tumours that produce PTHrP. The candidate marker would have to be detectable in biological fluids to be useful clinically.

PTHrP was also found to be strongly expressed by gastroenteropancreatic neuroendocrine tumours (chapter 3). If detected in the circulation in this group of patients, it may also be a potentially useful tumour marker. The glycoprotein chromogranin A (CgA), which is found in the storage granules of neuroendocrine cells and released during exocytosis, is currently used and is considered the most reliable serum marker for these tumours. Assays for its measurement in serum are established and widely available. Reported sensitivities for CgA in the detection of gastroenteropancreatic endocrine tumours are 84-92%.[ccxli, ccxlii] CgA sensitivity is higher for functioning compared to non-functioning tumours (96% versus 75%), and is higher in gastrointestinal versus pancreatic endocrine tumours (91% versus 74%).[ccxliv] Elevated levels of CgA are strongly correlated with tumour volume,[ccxliii] and are reliable markers in the follow-up of disease. Therefore there is less clinical need for novel tumour markers for these tumours. However used in combination with CgA, serum PTHrP may increase the sensitivity in the diagnosis of gastroenteropancreatic neuroendocrine tumours, particularly of non-functioning pancreatic endocrine tumours.

---

[3] SELDI technology is based on the selective retention of proteins on modified array surfaces, and requires relatively small amounts of material. Unbound proteins are discarded, and retained proteins are analysed by mass spectroscopy, generating a specific profile of the analysed proteome. Proteome profiles are then compared, and statistically significant differences in protein expression among different samples are deciphered. A major limitation of this technique is that individual proteins are not usually identified.

[4] Two protein microarray formats exist. The first involves high-density immobilisation arrays of antibodies. Bound proteins are detected by molecular tags. Limitations of this technique include the lack of availability of suitable binding molecules that can cope with protein diversity, and the fact that recognition molecules will profile binding epitopes rather than functional proteins, and may not discriminate between processed isoforms. A second format is reverse-phase protein microarray, where protein mixtures are arrayed and probed with a specific antibody, allowing semiquantification of the protein. Specific proteins (issued from complementary DNA expression libraries, phage display libraries, or protein fractionation) may be captured on the array, allowing the detection of autoantibodies in human serum.

For most patients with advanced hepatocellular carcinoma, pancreatic adenocarcinoma, or gastroenteropancreatic neuroendocrine tumours, treatment options are limited, resulting in a poor prognosis. Novel treatment strategies are therefore urgently required. A targeted therapy such as gene therapy is an example of a new strategy in cancer treatment. For gene therapy, delivery systems (viral or nonviral vectors) are used to introduce DNA contructs into living cells, in which the therapeutic gene products are expressed. For the treatment of malignant tumours, the reintroduction of tumour suppressor genes, expression of suicide genes rendering cells sensitive to chemotherapeutic agents, and expression of genes that block angiogenesis, are all possible approaches. However the problem lies in cell specific delivery of genes to tumour cells. Targeting gene therapy to tumour cells by the use of monoclonal antibodies that are specific to tumour cell surface antigens may result both in enhanced uptake of the delivery system and reduced toxicity, because targeted delivery systems will not damage normal cells not expressing the antigen. Antigen-specific antibodies may also be conjugated to chemotherapeutic agents such as methotrexate, and cytotoxic peptides to produce antitumour effects.[ccxliv] In this respect, PTHrP is an unsuitable target for this type of antibody-directed therapy, as it is also expressed by some normal tissues.

The development of humanized monoclonal antibodies against PTHrP may however have a role in the management of humoral hypercalcaemia of malignancy (HHM). A humanized antibody which specifically recognises PTHrP (1-34) and neutralizes PTHrP functions in vivo and vitro was administered intravenously into HHM mouse models bearing human pancreatic cancer and lung cancer xenografts. The human anti-PTHrP antibody normalized blood calcium levels. The animals also showed an improvement in body weight, food consumption, water intake, and renal function. These results demonstrated that PTHrP is a major pathogenic factor of hypercalcemia and cachexia in these animals, and suggested that the humanized anti-PTHrP antibody may be an effective and beneficial agent for patients with HHM.[ccxlv, ccxlvi]

Peptide receptor-targeted therapy is an alternative approach. This utilises knowledge of the expression of particular peptide receptors on tumour cells, and identifies small peptides that bind to active sites of the receptor. Random phage peptide display libraries have been widely used to identify such peptides that mimic epitopes

recognised by antibodies, and are now finding increasing application in the identification of small peptides that bind to active sites of other molecules, including receptors and enzymes. Such peptides may provide novel ligands for targeting tumour cells, and may be labelled with either diagnostic radionuclide tracers in order to accurately diagnose disease, or therapeutic radionuclide isotopes in order to treat disease. Alternatively these peptides could be linked to chemotherapeutic agents that are directly targeted to the tumour cells, or provide receptor antagonists, partial agonists, or agonists that down-regulate constitutive expression of the receptor.

In this study, PTHrP was localised to the nucleus in the following tumour cell lines: human prostatic adenocarcinoma PC3, human pancreatic adenocarcinoma BxPC3, rat buffalo hepatoma MCA RH 7777, and rat islet cell tumour CRI-G1. Furthermore, in PC3 cells, fluorescently labelled PTHrP (1-34) was internalised, transported and localized to the nucleus. Uptake of labelled peptide was attenuated by competition with unlabelled PTHrP (1-34), and therefore cellular uptake probably occurred through PTH1R-mediated endocytosis. Targeting PTH1R may therefore be an effective vehicle to the tumour cell cytoplasm and nucleus for the delivery of radionuclide isotopes, chemotherapy, or gene therapy. The expression of PTH1R by many normal tissues, however, as well as by tumour cells means that targeting this receptor with such agents is likely to cause intolerable toxicity. Another approach would be to deliver an agent that is expressed in normal cells, but is down-regulated by tumour cells. For example, the expression of a recently cloned β-galactosidase binding protein, galectin-8, whose function is to reduce cell migration, is decreased markedly during tumorigenesis in human colon tissue, and is expressed at a much lower level than in normal colonic tissue or in benign colonic tumours.[ccxlvii] Transfer of galectin-8 to tumour cells, may therefore be an appropriate therapeutic strategy to suppress tumour cell migration and metastases.

Alternatively, PTH1R isoforms may exist that are tumour specific, i.e. are not expressed by normal tissue. This phenomenon has been described for other G-protein-coupled peptide receptors such as the cholecystokinin 2 receptor (CCK-2R), whose natural ligand is gastrin, which is expressed by normal gastric glands and in wide spread areas of the brain, such as the cerebral cortex and limbic structures, and in gastrointestinal tumours such as colorectal cancers, pancreatic cancer and

hepatocellular carcinoma. A number of splice variants of the CCK-2R have been described, including a CCK-2R with a retained intron IV (CCK-2iIVsv). This splice variant, which appears to be constitutively active, is expressed very poorly in normal colorectal mucosa, but highly expressed in colorectal primary tumours and liver metastases. A stepwise increase in its expression is observed during the progression from premalignant Barrett's metaplasia to oesophageal carcinoma.[ccxlviii] The so-called 'truncated' PTH1R receptor detected as a 45kDa band by western immunoblotting of lysates from the prostate cancer cell line PC3, and the pancreatic adenocarcinoma cell lines, AR42J and Panc-1, may also represent a splice variant form of PTH1R. If this is a variant form of PTH1R, and is a functional receptor which is highly expressed by these tumour cells, but expressed at low levels, or not at all in normal cells, it may be a suitable target for receptor-targeted therapy of these tumours. This possibility should be further explored.

A third innovative avenue for targeting these resistant tumours is via immunotherapeutic strategies. Vaccines have been developed to target peptides involved in tumour autocrine pathways, such as the anti-gastrin vaccine, INSEGIA™ (Aphton Corporation, CA, USA), which neutralises amidated gastrin 17 and glycine-extended gastrin 17. Gastrin 17 is a growth factor for pancreatic, gastric and colorectal cancers. Phase 2 studies in patients with advanced pancreatic and gastric adenocarcinomas, have shown that the vaccine was well tolerated, produced adequate antibody responses in the majority of patients, and prolonged survival in pancreatic cancer patients.[ccxlix, ccl]

An autocrine pathway involving PTHrP/PTH1R has been documented in some tumour systems, where PTHrP has been shown to have a growth regulating effect. These studies have suggested that such a pathway also exists in adenocarcinoma of the pancreas, gastroenteropancreatic neuroendocrine tumours, and HCC. In gastroenteropancreatic neuroendocrine tumours, PTHrP may have an inhibitory effect on cell growth, and in HCC, PTHrP appears to promote cell growth. Therefore in HCC at least, PTHrP may be an appropriate target for this type of immunotherapy strategy, using a synthetic PTHrP peptide corresponding to the sequence involved in receptor binding and activation [PTHrP (1-34)] bound to a carrier molecule, to

generate an immune response against PTHrP, preventing its interaction with tumour cells. Such a strategy may also have a role in the management of patients with HHM.

An active immunisation approach has been used in patients in the treatment of hypercalcaemia due to parathyroid hormone secretion by parathyroid carcinoma. These tumours are highly differentiated and slow growing with a clinical course of years. Symptoms and death are the result of the metabolic actions of parathyroid hormone on PTH1R. Bradwell et al used human and bovine PTH peptide antigens synthesised on lysine webs as multiantigenic peptides mixed with Freund's complete adjuvant, in order to break self-tolerance (B-cell tolerance) and induce a strong immune response. An adequate antibody response to PTH was produced, with a corresponding reduction in serum calcium levels, accompanied by a marked clinical improvement.[cli] A successful anti-PTHrP vaccine may also have a beneficial effect against the symptoms of cancer-associated cachexia. PTHrP has recently been recognised as a procachectic factor; elevated serum levels of PTHrP are associated with the proinflammatory and cachectogenic serum cytokines tumour necrosis factor-alpha and interleukin-6.[cclii, ci]

This thesis has demonstrated that PTHrP and PTH1R are commonly expressed by tumour cells in human adenocarcinoma of the pancreas, gastroenteropancreatic neuroendocrine tumours, and hepatocellular carcinoma. The expression of both PTHrP protein and its corresponding receptor, PTH1R, suggests a possible autocrine/paracrine role for PTHrP/PTH1R in these tumours. In human HCC resection specimens, a positive correlation between PTHrP staining and staining for the nuclear proliferation antigen Ki67 was found, implying that PTHrP protein expression may influence, or be influenced by cellular proliferation in HCC. Furthermore, low level PTHrP staining was observed in normal liver resection specimens, followed by an observed sequential increase in PTHrP staining through cirrhotic liver, in which HCC normally arises, to the putative precursor lesions to HCC, macroregenerative nodules and dysplastic nodules, with a gradient of expression that peaked in malignant cells. This correlated with immunostaining for PTH1R across the tissue groups, supporting the existence of an autocrine/paracrine pathway for PTHrP/PTH1R in these tissues. Immunostaining for Ki67 was also

increased, from a low level of expression in cirrhotic liver and macroregenerative nodules, to an increased level in dysplastic nodules, with highest levels in HCC tumour cells. The gradient of PTHrP expression correlated Ki67 staining, suggesting that PTHrP, probably acting via PTH1R, is a likely candidate to influence hepatocyte and tumour cell growth.

If PTHrP, its isoforms, or cleavage products, are detectable in the circulation of patients with these tumours, they may be useful serum tumour markers. For pancreatic adenocarcinoma and HCC in particular, there is a clinical need for new biomarkers, in order to facilitate early tumour diagnosis at a stage when curative resection is possible, and to provide an effective screening tool in patients at high risk of developing these tumours. Once the pathophysiological role of PTHrP/PTH1R in human adenocarcinoma of the pancreas, hepatocellular carcinoma, and gastroenteropancreatic neuroendocrine tumours is better understood, agents that target this system, including novel receptor-targeted therapies and immunotherapeutic strategies, may have a therapeutic role in these often refractory tumours in which new agents for clinical evaluation remains a high priority.

# REFERENCES


[i] Wysolmerski JJ, Stewart AF. The physiology of parathyroid hormone-related protein: an emerging role as a developmental factor. Annu Rev Physiol 1998;60:431-460.
[ii] Karaplis AC, Luz A, Glowacki J, Bronson RT, Tybulewicz VL, Kronenberg HM, Mulligan RC. Lethal skeletal dysplasia from targeted disruption of the parathyroid hormone-related peptide gene. Genes Dev 1994;8:277-289.
[iii] Mallory TB. Case records of the Massachusetts General Hospital. Case #27461. N Engl J Med 1941;225:789-791.
[iv] Gellhorn A, Plimpton CH. Hypercalcemia in malignant disease without evidence of bone destruction. Am J Med 1956;21:750-759.
[v] Suva LJ, Winslow GA, Wettenhall RE, Hammonds RG, Moseley JM, Diefenbach-Jagger H, Rodda CP, Kemp BE, Rodriguez H, Chen EY. A parathyroid hormone-related protein implicated in malignant hypercalcemia: cloning and expression. Science 1987;237(4817):893-896.
[vi] Mangin M, Webb AC, Dreyer BE, Posillico JT, Ikeda K, Weir EC, Stewart AF, Bander NH, Milstone L, Barton DE. Identification of a cDNA encoding a parathyroid hormone-like peptide from a human tumor associated with humoral hypercalcemia of malignancy. Proc Natl Acad Sci U S A 1988;85(2):597-601.
[vii] Strewler GJ, Stern PH, Jacobs JW, Eveloff J, Klein RF, Leung SC, Rosenblatt M, Nissenson RA. Parathyroid hormonelike protein from human renal carcinoma cells. Structural and functional homology with parathyroid hormone. J Clin Invest 1987;80(6):1803-1807.
[viii] Mangin M, Ikeda K, Dreyer BE, Broadus AE. Isolation and characterization of the human parathyroid hormone-like peptide gene. Proc Natl Acad Sci U S A 1989;86(7):2408-2412.
[ix] Karaplis AC, Yasuda T, Hendy GN, Goltzman D, Banville D. Gene-encoding parathyroid hormone-like peptide: nucleotide sequence of the rat gene and comparison with the human homologue. Mol Endocrinol 1990;4(3):441-446.
[x] Mangin M, Ikeda K, Broadus AE. Structure of the mouse gene encoding parathyroid hormone-related peptide. Gene 1990;95(2):195-202.
[xi] Schermer DT, Chan SD, Bruce R, Nissenson RA, Wood WI, Strewler GJ. Chicken parathyroid hormone-related protein and its expression during embryologic development. J Bone Miner Res 1991;6(2):149-155.
[xii] Kukreja SC, Shevrin DH, Wimbiscus SA, Ebeling PR, Danks JA, Rodda CP, Wood WI, Martin TJ. Antibodies to parathyroid hormone-related protein lower serum calcium in athymic mouse models of malignancy-associated hypercalcemia due to human tumors. J Clin Invest 1988;82:1798-1802.
[xiii] Henderson J, Bernier S, D'Amour P, Goltzman D. Effects of passive immunization against parathyroid hormone (PTH)-like peptide and PTH in hypercalcemic tumor-bearing rats and normocalcemic controls. Endocrinology 1990;127:1310-1318.
[xiv] Wysolmerski JJ, Broadus AE. Hypercalcemia of malignancy: the central role of parathyroid hormone-related protein. Annu Rev Med 1994;45:189-200.
[xv] Rankin W, Grill V, Martin TJ. Parathyroid hormone-related protein and hypercalcemia. Cancer 1997;80(8 Suppl):1564-1571.
[xvi] Sellers RS, Luchin AI, Richard V, Brena RM, Lima D, Rosol TJ. Alternative splicing of parathyroid hormone-related protein mRNA: expression and stability. J Mol Endocrinol 2004;33(1):227-241.
[xvii] Lou H, Gagel RF. Alternative RNA processing--its role in regulating expression of calcitonin/calcitonin gene-related peptide. J Endocrinol 1998;156(3):401-405.
[xviii] Lou H, Gagel RF. Mechanism of tissue-specific alternative RNA processing of the calcitonin CGRP gene. Front Horm Res 1999;25:18-33.
[xix] Laugero LA, Oberbauer AM. Stability of alternatively spliced IGF-I mRNA in growth plate chondrocytes. Connect Tissue Res 2000;41(2):165-174.
[xx] Heath JK, Southby J, Fukumoto S, O'Keeffe LM, Martin TJ, Gillespie MT. Epidermal growth factor-stimulated parathyroid hormone-related protein expression involves increased gene transcription and mRNA stability. Biochem J 1995;307( Pt 1):159-167.
[xxi] Southby J, O'Keeffe LM, Martin TJ, Gillespie MT. Alternative promoter usage and mRNA splicing pathways for parathyroid hormone-related protein in normal tissues and tumours. Br J Cancer 1995;72(3):702-707.
[xxii] Werkmeister JR, Blomme EA, Weckmann MT, Grone A, McCauley LK, Wade AB, O'Rourke J, Capen CC, Rosol TJ. Effect of transforming growth factor-beta1 on parathyroid hormone-related protein secretion and mRNA expression by normal human keratinocytes in vitro. Endocrine 1998;8(3):291-299.

[xxiii] Guise TA, Yin JJ, Thomas RJ, Dallas M, Cui Y, Gillespie MT. Parathyroid hormone-related protein (PTHrP)-(1-139) isoform is efficiently secreted in vitro and enhances breast cancer metastasis to bone in vivo. Bone 2002;30(5):670-676.
[xxiv] Colasante A, Mascetra N, Brunetti M, Lattanzio G, Diodoro M, Caltagirone S, Musiani P, Aiello FB. Transforming growth factor beta 1, interleukin-8 and interleukin-1, in non-small-cell lung tumors. Am J Respir Crit Care Med 1997;156(3 Pt 1):968-973.
[xxv] Fiaschi-Taesch NM, Stewart AF. Minireview: parathyroid hormone-related protein as an intracrine factor--trafficking mechanisms and functional consequences. Endocrinology 2003;144(2):407-411.
[xxvi] Clemens TL, Cormier S, Eichinger A, Endlich K, Fiaschi-Taesch N, Fischer E, Friedman PA, Karaplis AC, Massfelder T, Rossert J, Schluter KD, Silve C, Stewart AF, Takane K, Helwig JJ. Parathyroid hormone-related protein and its receptors: nuclear functions and roles in the renal and cardiovascular systems, the placental trophoblasts and the pancreatic islets. Br J Pharmacol 2001;134(6):1113-1136.
[xxvii] Yang KH, dePapp AE, Soifer NE, Dreyer BE, Wu TL, Porter SE, Bellantoni M, Burtis WJ, Insogna KL, Broadus AE, et al. Parathyroid hormone-related protein: evidence for isoform- and tissue-specific posttranslational processing. Biochemistry 1994;33(23):7460-7469.
[xxviii] Goomer RS, Johnson KA, Burton DW, Amiel D, Maris TM, Gurjal A, Deftost LJ, Terkeltaub R. The tetrabasic KKKK(147-150) motif determines intracrine regulatory effects of PthrP 1-173 on chondrocyte PPi metabolism and matrix synthesis. Endocrinology 2000;141(12):4613-4622.
[xxix] Orloff JJ, Reddy D, de Papp AE, Yang KH, Soifer NE, Stewart AF. Parathyroid hormone-related protein as a prohormone: posttranslational processing and receptor interactions. Endocr Rev 1994;15(1):40-60.
[xxx] Fenton AJ, Kemp BE, Kent GN, Moseley JM, Zheng MH, Rowe DJ, Britto JM, Martin TJ, Nicholson GC. A carboxyl-terminal peptide from the parathyroid hormone-related protein inhibits bone resorption by osteoclasts. Endocrinology 1991;129(4):1762-1768.
[xxxi] Burtis WJ, Brady TG, Orloff JJ, Ersbak JB, Warrell RP Jr, Olson BR, Wu TL, Mitnick ME, Broadus AE, Stewart AF. Immunochemical characterization of circulating parathyroid hormone-related protein in patients with humoral hypercalcemia of cancer. N Engl J Med 1990;322(16):1106-1112.
[xxxii] Wu TL, Soifer NE, Burtis WJ, Milstone LM, Stewart AF. Glycosylation of parathyroid hormone-related peptide secreted by human epidermal keratinocytes. J Clin Endocrinol Metab 1991;73(5):1002-1007.
[xxxiii] Schluter K, Katzer C, Frischkopf K, Wenzel S, Taimor G, Piper HM. Expression, release, and biological activity of parathyroid hormone-related peptide from coronary endothelial cells. Circ Res 2000;86(9):946-951.
[xxxiv] Plawner LL, Philbrick WM, Burtis WJ, Broadus AE, Stewart AF. Cell type-specific secretion of parathyroid hormone-related protein via the regulated versus the constitutive secretory pathway. J Biol Chem 1995;270(23):14078-14084.
[xxxv] Abou-Samra AB, Juppner H, Force T, Freeman MW, Kong XF, Schipani E, Urena P, Richards J, Bonventre JV, Potts JT Jr, et al. Expression cloning of a common receptor for parathyroid hormone and parathyroid hormone-related peptide from rat osteoblast-like cells: a single receptor stimulates intracellular accumulation of both cAMP and inositol trisphosphates and increases intracellular free calcium. Proc Natl Acad Sci U S A 1992;89(7):2732-2736.
[xxxvi] McCuaig KA, Clarke JC, White JH. Molecular cloning of the gene encoding the mouse parathyroid hormone/parathyroid hormone-related peptide receptor. Proc Natl Acad Sci U S A 1994; 91(11): 5051–5055.
[xxxvii] Juppner H, Abou-Samra AB, Freeman M, Kong XF, Schipani E, Richards J, Kolakowski LF Jr, Hock J, Potts JT Jr, Kronenberg HM, et al. A G protein-linked receptor for parathyroid hormone and parathyroid hormone-related peptide. Science 1991;254(5034):1024-1026.
[xxxviii] Schipani E, Karga H, Karaplis AC, Potts JT Jr, Kronenberg HM, Segre GV, Abou-Samra AB, Juppner H. Identical complementary deoxyribonucleic acids encode a human renal and bone parathyroid hormone (PTH)/PTH-related peptide receptor. Endocrinology 1993;132(5):2157-2165.
[xxxix] Juppner H. Receptors for parathyroid hormone and parathyroid hormone-related peptide: exploration of their biological importance. Bone 1999;25(1):87-90.
[xl] Gether U. Uncovering molecular mechanisms involved in activation of G protein-coupled receptors. Endocr Rev 2000;21(1):90-113.
[xli] Kong XF, Schipani E, Lanske B, Joun H, Karperien M, Defize LH, Juppner H, Potts JT Jr, Segre GV, Kronenberg HM, et al. The rat, mouse, and human genes encoding the receptor for parathyroid

hormone and parathyroid hormone-related peptide are highly homologous. Biochem Biophys Res Commun 1994;201(2):1058.
[xlii] Joun H, Lanske B, Karperien M, Qian F, Defize L, Abou-Samra A. Tissue-specific transcription start sites and alternative splicing of the parathyroid hormone (PTH)/PTH-related peptide (PTHrP) receptor gene: a new PTH/PTHrP receptor splice variant that lacks the signal peptide. Endocrinology 1997;138(4):1742-1749.
[xliii] Vilardaga JP, Lin I, Nissenson RA. Analysis of Parathyroid Hormone (PTH)/Secretin Receptor Chimeras Differentiates the Role of Functional Domains in the PTH/ PTH-Related Peptide (PTHrP) Receptor on Hormone Binding and Receptor Activation Molecular Endocrinology 2001;15:1186-1199.
[xliv] Abou-Samra AB, Uneno S, Jueppner H, Keutmann H, Potts JT Jr, Segre GV, Nussbaum SR. Non-homologous sequences of parathyroid hormone and the parathyroid hormone related peptide bind to a common receptor on ROS 17/2.8 cells. Endocrinology 1989;125(4):2215-2217.
[xlv] Caulfield, M. P., and M. Rosenblatt. Parathyroid hormone-receptor interactions. Trends Endocrinol. Metab 1990;2:164-168.
[xlvi] Mannstadt M, Juppner H, Gardella TJ. Receptors for PTH and PTHrP: their biological importance and functional properties. Am J Physiol 1999;277(5 Pt 2):F665-675.
[xlvii] Mahon MJ, Cole JA, Lederer ED, Segre GV. Na+/H+ exchanger-regulatory factor 1 mediates inhibition of phosphate transport by parathyroid hormone and second messengers by acting at multiple sites in opossum kidney cells. Mol Endocrinol 2003;17(11):2355-2364.
[xlviii] Mahon MJ, Donowitz M, Segre GV. Direct binding of the PTH/PTHrP receptor (PTH1R) to the Na-H exchange regulatory factor 2 (NHERF2) selectively transfers signaling from adenylate cyclase to phospholipase C pathways. J Bone Miner Res 2001;16(suppl 1):S159.
[xlix] Whitfield JF, Isaacs RJ, Chakravarthy B, et al. Stimulation of protein kinase-C activity in cells expressing human parathyroid hormone (PTH) receptors by C- and N-terminally truncated fragments of PTH-(1-34). J Bone Miner Res 2001;16:441-447.
[l] Ferguson SS. Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. Pharmacol Rev 2001;53(1):1-24.
[li]Oakley RH, Laporte SA, Holt JA, Barak LS, Caron MG. Molecular determinants underlying the formation of stable intracellular G protein-coupled receptor-beta-arrestin complexes after receptor endocytosis. J Biol Chem 2001;276(22):19452-19460.
[lii] Sneddon WB, Magyar CE, Willick GE, Syme CA, Galbiati F, Bisello A, Friedman PA. Ligand-selective dissociation of activation and internalization of the parathyroid hormone (PTH) receptor: conditional efficacy of PTH peptide fragments. Endocrinology 2004;145(6):2815-2823.
[liii] Watson PH, Fraher LJ, Hendy GN, Chung UI, Kisiel M, Natale BV, Hodsman AB. Nuclear localization of the type 1 PTH/PTHrP receptor in rat tissues. J Bone Miner Res 2000;15(6):1033-1044.
[liv] Gaich G, Orloff JJ, Atillasoy EJ, Burtis WJ, Ganz MB, Stewart AF. Amino-terminal parathyroid hormone-related protein: specific binding and cytosolic calcium responses in rat insulinoma cells. Endocrinology 1993;132:1402-1409.
[lv] Orloff JJ, Kats Y, Urena P, Schipani E, Vasavada RC, Philbrick WM, Behal A, Abou-Samra AB, Segre GV, Juppner H. Further evidence for a novel receptor for amino-terminal parathyroid hormone-related protein on keratinocytes and squamous cell carcinoma cell lines. Endocrinology 1995;136(7):3016-3023.
[lvi] Yamamoto S, Morimoto I, Yanagihara N, Zeki K, Fujihira T, Izumi F, Yamashita H, Eto S. Parathyroid hormone-related peptide-(1-34) [PTHrP-(1-34)] induces vasopressin release from the rat supraoptic nucleus in vitro through a novel receptor distinct from a type I or type II PTH/PTHrP receptor. Endocrinology 1997;138(5):2066-2072.
[lvii] Rubin DA, Juppner H. Zebrafish express the common parathyroid hormone/parathyroid hormone-related peptide receptor (PTH1R) and a novel receptor (PTH3R) that is preferentially activated by mammalian and fugufish parathyroid hormone-related peptide. J Biol Chem 1999;274(40):28185-28190.
[lviii] Hoare SR, Rubin DA, Juppner H, Usdin TB. Evaluating the ligand specificity of zebrafish parathyroid hormone (PTH) receptors: comparison of PTH, PTH-related protein, and tuberoinfundibular peptide of 39 residues. Endocrinology 2000;141(9):3080-3086.
[lix] Robbins J, Dilworth SM, Laskey RA, Dingwall C. Two interdependent basic domains in nucleoplasmin nuclear targeting sequence: identification of a class of bipartite nuclear targeting sequence. Cell 1991;64(3):615-623.
[lx] Henderson JE, Amizuka N, Warshawsky H, Biasotto D, Lanske BM, Goltzman D, Karaplis AC. Nucleolar localization of parathyroid hormone-related peptide enhances survival of chondrocytes under conditions that promote apoptotic cell death. Mol Cell Biol 1995;15(8):4064-4075.

[lxi] de Miguel F, Fiaschi-Taesch N, Lopez-Talavera JC, Takane KK, Massfelder T, Helwig JJ, Stewart AF. The C-terminal region of PTHrP, in addition to the nuclear localization signal, is essential for the intracrine stimulation of proliferation in vascular smooth muscle cells. Endocrinology 2001;142(9):4096-4105.
[lxii] Huang Z, Chen Y, Nissenson RA. The cytoplasmic tail of the G-protein-coupled receptor for parathyroid hormone and parathyroid hormone-related protein contains positive and negative signals for endocytosis. J Biol Chem 1995;270(1):151-156.
[lxiii] Lam MH, House CM, Tiganis T, Mitchelhill KI, Sarcevic B, Cures A, Ramsay R, Kemp BE, Martin TJ, Gillespie MT. Phosphorylation at the cyclin-dependent kinases site (Thr85) of parathyroid hormone-related protein negatively regulates its nuclear localization. J Biol Chem 1999 ;274(26):18559-18566.
[lxiv] Amizuka N, Fukushi-Irie M, Sasaki T, Oda K, Ozawa H. Inefficient function of the signal sequence of PTHrP for targeting into the secretory pathway. Biochem Biophys Res Commun. 2000;273(2):621-629.
[lxv] Aarts MM, Rix A, Guo J, Bringhurst R, Henderson JE. The nucleolar targeting signal (NTS) of parathyroid hormone related protein mediates endocytosis and nucleolar translocation. J Bone Miner Res 1999;14(9):1493-1503.
[lxvi] Maher PA. Identification and characterization of a novel, intracellular isoform of fibroblast growth factor receptor-1(FGFR-1). J Cell Physiol 1996;169(2):380-390.
[lxvii] Nguyen MT, Karaplis AC. The nucleus: a target site for parathyroid hormone-related peptide (PTHrP) action. J Cell Biochem 1998;70(2):193-199.
[lxviii] Nguyen M, He B, Karaplis A. Nuclear forms of parathyroid hormone-related peptide are translated from non-AUG start sites downstream from the initiator methionine. Endocrinology 2001;142(2):694-703.
[lxix] Vagner S, Touriol C, Galy B, Audigier S, Gensac MC, Amalric F, Bayard F, Prats H, Prats AC. Translation of CUG- but not AUG-initiated forms of human fibroblast growth factor 2 is activated in transformed and stressed cells. J Cell Biol 1996;135(5):1391-1402.
[lxx] Kopito RR. ER quality control: the cytoplasmic connection. Cell. 1997 Feb 21;88(4):427-430.
[lxxi] Meerovitch K, Wing S, Goltzman D. Preproparathyroid hormone-related protein, a secreted peptide, is a substrate for the ubiquitin proteolytic system. J Biol Chem 1997;272(10):6706-6713.
[lxxii] Meerovitch K, Wing S, Goltzman D. Proparathyroid hormone-related protein is associated with the chaperone protein BiP and undergoes proteasome-mediated degradation. J Biol Chem 1998;273(33):21025-21030.
[lxxiii] Lam MH, Henderson B, Gillespie MT, Jans DA. Dynamics of leptomycin B-sensitive nucleocytoplasmic flux of parathyroid hormone-related protein. Traffic 2001;2(11):812-819.
[lxxiv] Lam MH, Thomas RJ, Loveland KL, Schilders S, Gu M, Martin TJ, Gillespie MT, Jans DA. Nuclear transport of parathyroid hormone (PTH)-related protein is dependent on microtubules. Mol Endocrinol 2002;16(2):390-401.
[lxxv] Lam MH, Briggs LJ, Hu W, Martin TJ, Gillespie MT, Jans DA. Importin beta recognizes parathyroid hormone-related protein with high affinity and mediates its nuclear import in the absence of importin alpha. J Biol Chem 1999;274(11):7391-7398.
[lxxvi]Lam MH, Olsen SL, Rankin WA, Ho PW, Martin TJ, Gillespie MT, Moseley JM. PTHrP and cell division: expression and localization of PTHrP in a keratinocyte cell line (HaCaT) during the cell cycle. J Cell Physiol 1997;173(3):433-446.
[lxxvii] Massfelder T, Dann P, Wu TL, Vasavada R, Helwig J-J, Stewart AF 1997 Opposing mitogenic and anti-mitogenic actions of parathyroid hormone-related protein in vascular smooth muscle cells: a critical role for nuclear targeting. Proc Natl Acad Sci U S A; 94:13630–13635.
[lxxviii] Aarts MM, Levy D, He B, Stregger S, Chen T, Richard S, Henderson JE. Parathyroid hormone-related protein interacts with RNA. J Biol Chem 1999;274:4832–4838
[lxxix]Tovar Sepulveda VA, Shen X, Falzon M. Intracrine PTHrP protects against serum starvation-induced apoptosis and regulates the cell cycle in MCF-7 breast cancer cells. Endocrinology 2002;143(2):596-606.
[lxxx] Gujral A, Burton DW, Terkeltaub R, Deftos LJ. Parathyroid hormone-related protein induces interleukin 8 production by prostate cancer cells via a novel intracrine mechanism not mediated by its classical nuclear localization sequence. Cancer Res 2001;61(5):2282-2288.
[lxxxi] Karaplis AC, Luz A, Glowacki J, Bronson RT, Tybulewicz VL, Kronenberg HM, Mulligan RC. Lethal skeletal dysplasia from targeted disruption of the parathyroid hormone-related peptide gene. Genes Dev 1994;8(3):277-289.

[lxxxii]Amizuka N, Warshawsky H, Henderson JE, Goltzman D, Karaplis AC. Parathyroid hormone-related peptide-depleted mice show abnormal epiphyseal cartilage development and altered endochondral bone formation. J Cell Biol 1994;126(6):1611-1623.
[lxxxiii] Lanske B, Karaplis AC, Lee K, Luz A, Vortkamp A, Pirro A, Karperien M, Defize LH, Ho C, Mulligan RC, Abou-Samra AB, Juppner H, Segre GV, Kronenberg HM. PTH/PTHrP receptor in early development and Indian hedgehog-regulated bone growth. Science 1996;273(5275):663-666.
[lxxxiv] Qian J, Colbert MC, Witte D, Kuan CY, Gruenstein E, Osinska H, Lanske B, Kronenberg HM, Clemens TL. Midgestational lethality in mice lacking the parathyroid hormone (PTH)/PTH-related peptide receptor is associated with abrupt cardiomyocyte death. Endocrinology 2003;144(3):1053-1061.
[lxxxv] Boabaid F, Berry JE, Koh AJ, Somerman MJ, McCcauley LK. The role of parathyroid hormone-related protein in the regulation of osteoclastogenesis by cementoblasts. J Periodontol. 2004;75(9):1247-1254.
[lxxxvi] Suda T, Takahashi N, Udagawa N, Jimi E, Gillespie MT, Martin TJ. Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. Endocr Rev 1999;20(3):345-357.
[lxxxvii] Danks JA, Ebeling PR, Hayman J, Chou ST, Moseley JM, Dunlop J, Kemp BE, Martin TJ. Parathyroid hormone-related protein: immunohistochemical localization in cancers and in normal skin. J Bone Miner Res 1989;4(2):273-278.
[lxxxviii] Kageshita T, Matsui T, Hirai S, Fukuda Y, Ono T. Hypercalcaemia in melanoma patients associated with increased levels of parathyroid hormone-related protein. Melanoma Res 1999;9(1):69-73.
[lxxxix] Cisneros G, Lara LF, Crock R, Whittier FC. Humoral hypercalcemia of malignancy in squamous cell carcinoma of the skin: parathyroid hormone-related protein as a cause. South Med J 2001;94(3):329-331.
[xc] Southby J, Kissin MW, Danks JA, Hayman JA, Moseley JM, Henderson MA, Bennett RC, Martin TJ. Immunohistochemical localization of parathyroid hormone-related protein in human breast cancer. Cancer Res 1990;50(23):7710-7716.
[xci] Powell GJ, Southby J, Danks JA, Stillwell RG, Hayman JA, Henderson MA, Bennett RC, Martin TJ. Localization of parathyroid hormone-related protein in breast cancer metastases: increased incidence in bone compared with other sites. Cancer Res 1991;51(11):3059-3061.
[xcii] Vargas SJ, Gillespie MT, Powell GJ, Southby J, Danks JA, Moseley JM, Martin TJ. Localization of parathyroid hormone-related protein mRNA expression in breast cancer and metastatic lesions by in situ hybridization. J Bone Miner Res 1992;7(8):971-979.
[xciii] Gotoh A, Kitazawa S, Mizuno Y, Takenaka A, Arakawa S, Matsumoto O, Kitazawa R, Fujimori T, Maeda S, Kamidono S. Common expression of parathyroid hormone-related protein and no correlation of calcium level in renal cell carcinomas. Cancer 1993;71(9):2803-2806.
[xciv] Iwamura M, Wu W, Muramoto M, Ohori M, Egawa S, Uchida T, Baba S. Parathyroid hormone-related protein is an independent prognostic factor for renal cell carcinoma. Cancer 1999;86(6):1028-1034.
[xcv] Massfelder T, Lang H, Schordan E, Lindner V, Rothhut S, Welsch S, Simon-Assmann P, Barthelmebs M, Jacqmin D, Helwig JJ. Parathyroid hormone-related protein is an essential growth factor for human clear cell renal carcinoma and a target for the von Hippel-Lindau tumor suppressor gene. Cancer Res 2004;64(1):180-188.
[xcvi] Davidson LA, Black M, Carey FA, Logue F, McNicol AM. Lung tumours immunoreactive for parathyroid hormone related peptide: analysis of serum calcium levels and tumour type. J Pathol 1996;178(4):398-401.
[xcvii] Hastings RH, Araiza F, Burton DW, Deftos LJ. Role of parathyroid hormone-related protein in lung cancer cell survival. Chest 2004;125(5 Suppl):150S.
[xcviii] Hiraki A, Ueoka H, Bessho A, Segawa Y, Takigawa N, Kiura K, Eguchi K, Yoneda T, Tanimoto M, Harada M. Parathyroid hormone-related protein measured at the time of first visit is an indicator of bone metastases and survival in lung carcinoma patients with hypercalcemia. Cancer 2002;95(8):1706-1713.
[xcix] Malakouti S, Asadi FK, Kukreja SC, Abcarian HA, Cintron JR. Parathyroid hormone-related protein expression in the human colon: immunohistochemical evaluation. Am Surg 1996;62(7):540-544.
[c] Alipov GK, Ito M, Nakashima M, Ikeda Y, Nakayama T, Ohtsuru A, Yamashita S, Sekine I. Expression of parathyroid hormone-related peptide (PTHrP) in gastric tumours. J Pathol 1997;182(2):174-179.

[ci] Deans C, Wigmore S, Paterson-Brown S, Black J, Ross J, Fearon KC. Serum parathyroid hormone-related peptide is associated with systemic inflammation and adverse prognosis in gastroesophageal carcinoma. Cancer 2005;103(9):1810-1818.
[cii] Zondek H, Petow H, Siebert W. Die bedeutung der calciumbestimmung im blute fur die diagnose der niereninsuffizienz. Zertsch Klin Med 1923;99:129-138.
[ciii] Yamada S, Sanefuji H, Morimoto H, Harada Y, Mine S, Morimoto I, Eto S. Parathyroid hormone-related peptide producing cholangiocellular carcinoma with a marked psammoma formation. J Gastroenterol Hepatol 2000;15:1442-1446.
[civ] Bouvet M, Nardin SR, Burton DW, Behling C, Carethers JM, Moossa AR, Deftos LJ. Human pancreatic adenocarcinomas express parathyroid hormone-related protein. J Clin Endocrinol & Metabol 2001;86:310-316.
[cv] Asa SL, Henderson J, Goltzman D, Drucker DJ. Parathyroid hormone-like peptide in normal and neoplastic human endocrine tissues. J Clin Endocrinol Metab 1990;71(5):1112-1118.
[cvi] Drucker DJ, Asa SL, Henderson J, Goltzman D. The parathyroid hormone-like peptide gene is expressed in the normal and neoplastic human endocrine pancreas. Mol Endocrinol 1989;3:1589-1595.
[cvii] Ito M, Enomoto H, Usa T, Villadolid MC, Ohtsuru A, Namba H, Sekine I, Yamashita S. Expression of parathyroid hormone related peptide in human pituitary tumours. J Clin Pathol 1993;46(7):682-683.
[cviii] Kramer S, Reynolds FH Jr, Castillo M, Valenzuela DM, Thorikay M, Sorvillo JM. Immunological identification and distribution of parathyroid hormone-like protein polypeptides in normal and malignant tissues. Endocrinology 1991;128(4):1927-1937.
[cix]Deftos LJ, Burton DW, Brandt DW, Pinar H, Rubin LP. Neoplastic hormone-producing cells of the placenta produce and secrete parathyroid hormone-related protein. Studies by immunohistology, immunoassay, and polymerase chain reaction. Lab Invest 1994;71(6):847-852.
[cx] Stewart AF, Romero R, Schwartz PE, Kohorn EI, Broadus AE. Hypercalcemia associated with gynecologic malignancies: biochemical characterization. Cancer 1982;49(11):2389-2394.
[cxi] Kitazawa S, Kitazawa R, Fukase M, Fujimori T, Maeda S. Immunohistochemical evaluation of parathyroid hormone-related protein (PTHrP) in the uterine cervix. Int J Cancer 1992;50(5):731-735.
[cxii] Burton PB, Knight DE, Quirke P, Smith R, Moniz C. Parathyroid hormone related peptide in ovarian carcinoma. J Clin Pathol 1990;43(9):784.
[cxiii] Matias-Guiu X, Prat J, Young RH, Capen CC, Rosol TJ, Delellis RA, Scully RE. Human parathyroid hormone-related protein in ovarian small cell carcinoma. An immunohistochemical study. Cancer 1994 ;73(7):1878-1881.
[cxiv] Deftos LJ, Barken I, Burton DW, Hoffman RM, Geller J. Direct evidence that PTHrP expression promotes prostate cancer progression in bone. Biochem Biophys Res Commun. 2005;327(2):468-472.
[cxv] Asadi F, Kukreja S. Parathyroid hormone-related protein in prostate cancer. Crit Rev Eukaryot Gene Expr 2005;15(1):15-28.
[cxvi] Evliyaoglu C, Carroll R, Folkerth R, Bello L, Bruns DE, Black PM. Parathyroid hormone-related protein and its receptor in human glial tumors. Acta Neurochir (Wien) 2000;142(8):871-878.
[cxvii] Chattopadhyay N, Evliyaoglu C, Heese O, Carroll R, Sanders J, Black P, Brown EM. Regulation of secretion of PTHrP by Ca(2+)-sensing receptor in human astrocytes, astrocytomas, and meningiomas. Am J Physiol Cell Physiol 2000;279(3):C691-699.
[cxviii] Pardo FS, Lien WW, Fox HS, Efird JT, Aguilera JA, Burton DW, Deftos LJ. Parathyroid hormone-related protein expression is correlated with clinical course in patients with glial tumors. Cancer 2004 ;101(11):2622-2628.
[cxix] Ikeda K, Ohno H, Hane M, Yokoi H, Okada M, Honma T, Yamada A, Tatsumi Y, Tanaka T, Saitoh T, et al. Development of a sensitive two-site immunoradiometric assay for parathyroid hormone-related peptide: evidence for elevated levels in plasma from patients with adult T-cell leukemia/lymphoma and B-cell lymphoma. J Clin Endocrinol Metab 1994;79(5):1322-1327.
[cxx] Kitazawa R, Kitazawa S, Kajimoto K, Sowa H, Sugimoto T, Matsui T, Chihara K, Maeda S. Expression of parathyroid hormone-related protein (PTHrP) in multiple myeloma. Pathol Int. 2002;52(1):63-68.
[cxxi] McStay M, Savage K, Stubbs M, Khan K, Dhillon AP, Caplin ME. Expression and uptake of parathyroid hormone related peptide in hepatocellular carcinoma. Gut 2003; 52(Suppl 1): A336.
[cxxii] Vasavada RC, Cavaliere C, D'Ercole AJ, Dann P, Burtis WJ, Madlener AL, et al. Overexpression of parathyroid hormone-related protein in the pancreatic islets of transgenic mice causes islet hyperplasia, hyperinsulinaemia , and hypoglycaemia. J Biol Chem 1996;271:1200-1208.

[cxxiii] Porter SE, Sorenson RL, Dann P, Garcia-Ocana A, Stewart AF, Vasavada RC. Progressive pancreatic islet hyperplasia in the islet-targeted parathyroid hormone–related protein over-expressing mouse. Endocrinology 1998;139:3743-3751.
[cxxiv] Mitlak BH, Hutchinson JS, Kaufman SD, Nussman SR. Parathyroid hormone-related peptide mediates hypercalcaemia in an islet cell tumour of the pancreas. Horm Metab Res 1991;23:344-346.
[cxxv] Mao C, Carter P, Schaefer P, et al. Malignant islet cell tumour associated with hypercalcaemia. Surgery 1995;117:37-40.
[cxxvi] Williams EJ, Ratcliffe WA, Stavri GT, Stamatakis JD. Hypercalcaemia secondary to secretion of parathyroid hormone related protein from a somatostatinoma of the pancreas. Ann Clin Biochem 1992;29:354-357.
[cxxvii] Rizzoli R, Sappino AP, Bonjour JP. Parathyroid hormone-related protein and hypercalcaemia in pancreatic neuroendocrine tumours. Int J Cancer 1990;46:394-398.
[cxxviii] Jensen RT, Norton JA. Endocrine tumours of the Pancreas. *In* Feldman M, Scharschmidt BF, Sleisenger MH (Eds). Gastrointestinal and Liver Disease: Pathophysiology/ Diagnosis/ Management, 6th Ed. Philadelphia, W.B.Saunders Co.,1998 p.884.
[cxxix] Miraliakbari BA, Asa SL, Boudreau SF. Parathyroid hormone-like peptide in pancreatic endocrine carcinoma and adenocarcinoma associated with hypercalcaemia. Hum Pathol 1992;23(8):884-887.
[cxxx] Tachibana I, Nakano S, Akiyama T, Ogami Y, Tabaru A, Otsuki M. Parathyroid hormone-related protein mediates hypercalcaemia in an exocrine pancreatic cancer. Am J Gastroenterol 1994;89:1580-1581.
[cxxxi] Kakizaki S, Ohya N, Yoshinaga T, Higuchi T, Kitazawa R, Takayama H, Takagi H, Nagamine T, Mori M. Undifferentiated pancreatic cancer associated with humoral hypercalcemia of malignancy. Jpn J Clin Oncol 1998;28:563-566.
[cxxxii] Roskams T, Campos RV, Drucker DJ, Desmet VJ. Reactive human bile ductules express parathyroid hormone-related peptide. Histopathology 1993;23(1):11-19.
[cxxxiii] Downey SE, Hoyland J, Freemont AJ, Knox F, Walls J, Bundred NJ. Expression of the receptor for parathyroid hormone-related protein in normal and malignant breast tissue. J Pathol 1997;183(2):212-217.
[cxxxiv] Iezzoni JC, Bruns ME, Frierson HF, Scott MG, Pence RA, Deftos LJ, Bruns DE. Coexpression of parathyroid hormone-related protein and its receptor in breast carcinoma: a potential autocrine effector system. Mod Pathol 1998;11(3):265-270.
[cxxxv] Sipos B, Moser S, Kalthoff H, Torok V, Lohr M, Kloppel G. A comprehensive characterization of pancreatic ductal carcinoma cell lines: towards the establishment of an in vitro research platform. Virchows Arch 2003;442:444-452.
[cxxxvi] Dickinson CJ, Daugherty D, Guo YJ, Hughes P, Yamada T. Molecular analysis of dibasic endoproteolytic cleavage signals. J Biol Chem 1992;267:21795–21801.
[cxxxvii] Iwamura M, Abrahamsson PA, Foss KA, Wu G, Cockett AT, Deftos LJ. Parathyroid hormone-related protein: a potential autocrine growth regulator in human prostate cancer cell lines. Urology 1994;43(5):675-679.
[cxxxviii] Tovar Sepulveda VA, Falzon M. Parathyroid hormone-related protein enhances PC-3 prostate cancer cell growth via both autocrine/paracrine and intracrine pathways. Regul Pept 2002;105:109-120.
[cxxxix] Iddon J, Bundred NJ, Hoyland J, Downey SE, Baird P, Salter D, McMahon R, Freemont AJ. Expression of parathyroid hormone-related protein and its receptor in bone metastases from prostate cancer. J Pathol 2000;191:170-174.
[cxl] Bui TD, Shallal A, Malik AN, al-Mahdawi S, Moscoso G, Bailey ME, Burton PB, Moniz C. Parathyroid hormone related peptide gene expression in human fetal and adult heart. Cardiovasc Res 1993;27:1204-1208.
[cxli] Zhao Q, Brauer PR, Xiao L, McGuire MH, Yee JA. Expression of parathyroid hormone-related peptide (PthrP) and its receptor (PTH1R) during the histogenesis of cartilage and bone in the chicken mandibular process. J Anat 2002;201:137-151.
[cxlii] Schluter K, Katzer C, Frischkopf K, Wenzel S, Taimor G, Piper HM. Expression, release, and biological activity of parathyroid hormone-related peptide from coronary endothelial cells. Circ Res 2000;86(9):946-951.
[cxliii] Bisello A, Greenberg Z, Behar V, Rosenblatt M, Suva LJ, Chorev M. Role of glycosylation in expression and function of the human parathyroid hormone/parathyroid hormone-related protein receptor. Biochemistry 1996;35:15890-15895.
[cxliv] Leotlela PD, Jauch A, Holtgreve-Grez H, Thakker RV. Genetics of neuroendocrine and carcinoid tumours. Endocr Relat Cancer 2003;10:437-450.

[cxlv] McStay M, Caplin ME. GI Hormone Producing Tumours: Syndromes and Treatment Options. In Johnson CD and Imrie CW (eds), Pancreatic Disease: Basic Science and Clinical Management. Springer-Verlag London Limited 2004, 2003, pp31-53.
[cxlvi] McStay MK, Caplin ME. Carcinoid tumour. Minerva Med 2002;93:389-401.
[cxlvii] Barhoum M, Hutchins L, Fonseca VA. Intractable hypercalcemia due to a metastatic carcinoid secreting parathyroid hormone-related peptide and interleukin-6: response to octreotide. Am J Med Sci 1999;318:203-205.
[cxlviii] Mantzoros CS, Suva LJ, Moses AC, Spark R. Intractable hypercalcaemia due to parathyroid hormone-related peptide secretion by a carcinoid tumour. Clin Endocrinol (Oxf) 1997;46(3):373-375.
[cxlix] Gerdes J, Lemke H, Baisch H, Wacker H-H, Schwab U, Stein U, Stein H. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. J Immunol 1984;133:1710-1715.
[cl] Cattoretti G, Becker MH, Key G, Duchrow M, Schluter C, Galle J, Gerdes J. Monoclonal antibodies against recombinant parts of the Ki-67 antigen (MIB 1 and MIB 3) detect proliferating cells in microwave-processed formalin-fixed paraffin sections.
J Pathol 1992;168:357-363.
[cli] Waldherr C, Pless M, Maecke HR, et al. Tumor response and clinical benefit in neuroendocrine after 7.4 GBq $^{90}$Y-DOTATOC. J Nucl Med 2002;43:610-616.
[cliii] Virgolini I, Britton K, Buscombe JR, Moncayo R, Paganelli G, Riva P. In- and Y-DOTA-lanreotide: results and implications of the MAURITIUS trial. Semin Nucl Med 2002;32(2):148-155.
[cliii] Teunissen JJ, Kwekkeboom DJ, Krenning EP. Quality of life in patients with gastroenteropancreatic tumors treated with [177Lu-DOTA0,Tyr3]octreotate. J Clin Oncol 2004;22(13):2724-2729.
[cliv] Watson PH, Fraher LJ, Natale BV, Kisiel M, Hendy GN, Hodsman AB. Nuclear localization of the type 1 parathyroid hormone/parathyroid hormone-related peptide receptor in MC3T3-E1 cells: association with serum-induced cell proliferation. Bone 2000;26(3):221-225.
[clv] Li H, Seitz PK, Selvanayagam P, Rajaraman S, Cooper CW. Effect of endogenously produced parathyroid hormone-related peptide on growth of a human hepatoma cell line (Hep G2). Endocrinology 1996;137:2367-2374.
[clvi] Yen TC, Hwang SJ, Wang CC, Lee SD, Yeh SH. Hypercalcemia and parathyroid hormone-related protein in hepatocellular carcinoma. Liver 1993;13:311-5.
[clvii] Oldenburg WA, van Heerden JA, Sizemore GW, Abboud CF, Sheedy PF 2nd. Hypercalcemia and primary hepatic tumors. Arch Surg 1982;117:1363-1366.
[clviii] Keller RT, Goldschneider I, Lafferty FW. Hypercalcaemia secondary to a primary hepatoma. J Am Med Assoc 1965;192:782-784.
[clix] Roskams T, Willems M, Campos RV, Drucker DJ, Yap SH, Desmet VJ. Parathyroid hormone-related peptide expression in primary and metastatic liver tumours. Histopathology 1993;23:519-525.
[clx] Yamada S, Sanefuji H, Morimoto H, Harada Y, Mine S, Morimoto I, Eto S. Parathyroid hormone-related peptide producing cholangiocellular carcinoma with a marked psammoma formation. J Gastroenterol Hepatol 2000;15:1442-1446.
[clxi] Roskams T, Moshage H, Depla E, Willems M, Desmet V, Yap P. Parathyroid hormone-related peptide is expressed and rapidly inducible in human liver cell cultures that have a bile duct phenotype. J Hepatol 1995;23:160-165.
[clxii] Naide W, Matz R, Spear PW. Cholangiocarcinoma causing hypercalcemia and hypophosphatemia without skeletal metastases (pseudohyperparathyroidism). Am J Dig Dis 1968;13:705-708.
[clxiii]Davis JM, Sadasivan R, Dwyer T, Van Veldhuizen P. Case report: cholangiocarcinoma and hypercalcemia. Am J Med Sci 1994;307:350-352.
[clxiv] Lee JK, Chuang MJ, Lu CC, Hao LJ, Yang CY, Han TM, Lam HC. Parathyroid hormone and parathyroid hormone related protein assays in the investigation of hypercalcemic patients in hospital in a Chinese population. J Endocrinol Invest 1997;20:404-409.
[clxv] Karpf DB, Arnaud CD, King K, Bambino T, Winer J, Nyiredy K, Nissenson RA. The canine renal parathyroid hormone receptor is a glycoprotein: characterization and partial purification. Biochemistry 1987;26:7825-7833.
[clxvi] Okuda K, Fujimoto I, Hanai A, Urano Y. Changing incidence of hepatocellular carcinoma in Japan. Cancer Res 1987;47:4967-4972.
[clxvii] El-Serag HB, Mason AC. Rising incidence of hepatocellular carcinoma in the United States. N Engl J Med 1999;340:745-750.
[clxviii] Taylor-Robinson SD, Foster GR, Arora S, Hargreaves S, Thomas HC.Increase in primary liver cancer in the UK, 1979-94. Lancet 1997;350(9085):1142-1143.
[clxix] Okuda K. Hepatocellular carcinoma. J Hepatol 2000;32(1 Suppl):225-237.

[clxx] Campos RV, ASA SL, Drucker DJ. Immunocytochemical localization of parathyroid hormone-like peptide in the rat fetus. Cancer Res 1991;51:6351-6357.
[clxxi] Moniz C, Burton PB, Malik AN, Dixit M, Banga JP, Nicolaides K, Quirke P, Knight DE, McGregor AM. Parathyroid hormone-related peptide in normal human fetal development. J Mol Endocrinol 1990;5:259-266.
[clxxii] Ikeda K, Weir EC, Mangin M, Dannies PS, Kinder B, Deftos LJ, Brown EM, Broadus AE. Expression of messenger ribonucleic acids encoding a parathyroid hormone-like peptide in normal human and animal tissues with abnormal expression in human parathyroid adenomas. Mol Endocrinol 1988;2:1230-1236.
[clxxiii] Ikeda K, Mangin M, Dreyer BE, Webb AC, Posillico JT, Stewart AF, Bander NH, Weir EC, Insogna KL, Broadus AE. Identification of transcripts encoding a parathyroid hormone-like peptide in messenger RNAs from a variety of human and animal tumors associated with humoral hypercalcemia of malignancy. J Clin Invest 1988;81:2010-2014.
[clxxiv] Funk JL, Moser AH, Grunfeld C, Feingold KR. Parathyroid hormone-related protein is induced in the adult liver during endotoxemia and stimulates the hepatic acute phase response. Endocrinology 1997;138:2665-2673.
[clxxv] Urena P, Kong XF, Abou-Samra AB, Juppner H, Kronenberg HM, Potts JT Jr, Segre GV. Parathyroid hormone (PTH)/PTH-related peptide receptor messenger ribonucleic acids are widely distributed in rat tissues. Endocrinology 1993;133:617-623.
[clxxvi] Tian J, Smogorzewski M, Kedes L, Massry SG. Parathyroid hormone-parathyroid hormone related protein receptor messenger RNA is present in many tissues besides the kidney. Am J Nephrol 1993;13:210-213.
[clxxvii] Bergeron JJ, Tchervenkov S, Rouleau MF, Rosenblatt M, Goltzman D. In vivo demonstration of receptors in rat liver to the amino-terminal region of parathyroid hormone. Endocrinology 1981;109:1552-1559.
[clxxviii] Neuman WF, Schneider N. The parathyroid hormone-sensitive adenylate cyclase system in plasma membranes of rat liver. Endocrinology 1980;107:2082-2087.
[clxxix] Canterbury JM, Levy G, Ruiz E, Reiss E. Parathyroid hormone activation of adenylate cyclase in liver. Proc Soc Exp Biol Med 1974;147:366-370.
[clxxx] Mine T, Kojima I, Ogata E. Calcium rather than cyclic AMP is an intracellular messenger of parathyroid hormone action on glycogen metabolism in isolated rat hepatocytes. Biochem J 1989;258:889-894.
[clxxxi] Klin M, Smogorzewski M, Khilnani H, Michnowska M, Massry SG. Mechanisms of PTH-induced rise in cytosolic calcium in adult rat hepatocytes. Am J Physiol 1994;267:G754-763.
[clxxxii] Coxam V, Davicco MJ, Durand D, Bauchart D, Lefaivre J, Barlet JP. The influence of parathyroid hormone-related protein on hepatic IGF-1 production. Acta Endocrinol 1992;126:430-433.
[clxxxiii] Moxley MA, Bell NH, Wagle SR, Allen DO, Ashmore J. Parathyroid hormone stimulation of glucose and urea production in isolated liver cells. Am J Physiol 1974;227:1058-1061.
[clxxxiv] Hems DA, Harmon CS, Whitton PD. Inhibition by parathyroid hormone of glycogen synthesis in the perfused rat liver. FEBS Lett. 1975;58:167-169.
[clxxxv] Hruska KA, Blondin J, Bass R, Santiago J, Thomas L, Altsheler P, Martin K, Klahr S. Effect of intact parathyroid hormone on hepatic glucose release in the dog. J Clin Invest 1979;64:1016-1023.
[clxxxvi] Arakawa M, Kage M, Sugihara S, Nakashima T, Suenaga M, Okuda K. Emergence of malignant lesions within an adenomatous hyperplastic nodule in a cirrhotic liver. Observations in five cases. Gastroenterology 1986;91:198-208.
[clxxxvii] Ueda K, Terada T, Nakanuma Y, Matsui O. Vascular supply in adenomatous hyperplasia of the liver and hepatocellular carcinoma: a morphometric study. Hum Pathol 1992;23:619-626
[clxxxviii] Matsui O, Kadoya M, Kameyama T, Yoshikawa J, Takashima T, Nakanuma Y, Unoura M, Kobayashi K, Izumi R, Ida M, et al. Benign and malignant nodules in cirrhotic livers: distinction based on blood supply. Radiology 1991;178:493-497.
[clxxxix] Takayama T, Makuuchi M, Hirohashi S, Sakamoto M, Okazaki N, Takayasu K, Kosuge T, Motoo Y, Yamazaki S, Hasegawa H. Malignant transformation of adenomatous hyperplasia to hepatocellular carcinoma. Lancet 1990;336:1150-1153.
[cxc] Eguchi A, Nakashima O, Okudaira S, Sugihara S, Kojiro M. Adenomatous hyperplasia in the vicinity of small hepatocellular carcinoma. Hepatology 1992;15(5):843-848.
[cxci] Theise ND. Cirrhosis and hepatocellular neoplasia: more like cousins than like parent and child. Gastroenterology 1996;111:526-528.
[cxcii] Iwamura M, Wu G, Abrahamsson PA, di Sant'Agnese PA, Cockett AT, Deftos LJ. Parathyroid hormone-related protein is expressed by prostatic neuroendocrine cells. Urology 1994;43:667-674.

[cxciii] Cramer SD, Peehl DM, Edgar MG, Wong ST, Deftos LJ, Feldman D. Parathyroid hormone--related protein (PTHrP) is an epidermal growth factor-regulated secretory product of human prostatic epithelial cells. Prostate 1996;29:20-29.
[cxciv] Iwamura M, Gershagen S, Lapets O, Moynes R, Abrahamsson PA, Cockett AT, Deftos LJ, di Sant'Agnese PA. Immunohistochemical localization of parathyroid hormone-related protein in prostatic intraepithelial neoplasia. Hum Pathol 1995;26:797-801.
[cxcv] Iwamura M, di Sant'Agnese PA, Wu G, Benning CM, Cockett AT, Deftos LJ, Abrahamsson PA. Immunohistochemical localization of parathyroid hormone-related protein in human prostate cancer. Cancer Res 1993;53:1724-1726.
[cxcvi] Wu G, Iwamura M, di Sant'Agnese PA, Deftos LJ, Cockett AT, Gershagen S. Characterization of the cell-specific expression of parathyroid hormone-related protein in normal and neoplastic prostate tissue. Urology 1998;51:110-120.
[cxcvii] Asadi F, Farraj M, Sharifi R, Malakouti S, Antar S, Kukreja S. Enhanced expression of parathyroid hormone-related protein in prostate cancer as compared with benign prostatic hyperplasia. Hum Pathol 1996;27(12):1319-1323.
[cxcviii] Burton DW, Deftos LJ. Co-expression of the PTHrP receptor and PTHrP peptides in human prostate cells. Proceedings of the 81st Annual Meeting of the Endocrine Society, San Diego, California, June 12-15, 1999.
[cxcix] Dougherty KM, Blomme EA, Koh AJ, Henderson JE, Pienta KJ, Rosol TJ, McCauley LK. Parathyroid hormone-related protein as a growth regulator of prostate carcinoma. Cancer Res. 1999;59:6015-6022.
[cc] Tiniakos DG, Brunt EM. Proliferating cell nuclear antigen and Ki-67 labeling in hepatocellular nodules: a comparative study. Liver 1999;19:58-68.
[cci] Theise ND, Marcelin K, Goldfischer M, Hytiroglou P, Ferrell L, Thung SN. Low proliferative activity in macroregenerative nodules: evidence for an alternate hypothesis concerning human hepatocarcinogenesis. Liver 1996;16:134-139.
[ccii] Park YN, Kim YB, Yang KM, Park C. Increased expression of vascular endothelial growth factor and angiogenesis in the early stage of multistep hepatocarcinogenesis. Arch Pathol Lab Med 2000;124:1061-1065.
[cciii] Farber E. Hepatocyte proliferation in stepwise development of experimental liver cell cancer. Dig Dis Sci 1991;36:973-978.
[cciv] Borzio M, Trere D, Borzio F, Ferrari AR, Bruno S, Roncalli M, Colloredo G, Leandro G, Oliveri F, Derenzini M. Hepatocyte proliferation rate is a powerful parameter for predicting hepatocellular carcinoma development in liver cirrhosis. Mol Pathol 1998;51:96-101.
[ccv] Donato MF, Arosio E, Del Ninno E, Ronchi G, Lampertico P, Morabito A, Balestrieri MR, Colombo M. High rates of hepatocellular carcinoma in cirrhotic patients with high liver cell proliferative activity. Hepatology 2001;34:523-528.
[ccvi] Knowles BB, Howe CC, Aden DP. Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. Science 1980;209:497-499.
[ccvii] Javitt NB. Hep G2 cells as a resource for metabolic studies: lipoprotein, cholesterol, and bile acids. FASEB J 1990;4:161-168.
[ccviii] Falzon M, Du P. Enhanced growth of MCF-7 breast cancer cells overexpressiong parathyroid hormone-related peptide. Endocrinology 2000;141(5):1882-1892.
[ccix] Massfelder T, Taesch N, Endlich N, Eichinger A, Escande B, Endlich K, Barthelmebs M, Helwig JJ. Paradoxical actions of exogenous and endogenous parathyroid hormone-related protein on renal vascular smooth muscle cell proliferation: reversion in the SHR model of genetic hypertension. FASEB J 2001;15:707-718.
[ccx] Abou-Samra AB, Jueppner H, Potts JT Jr, Segre GV. Abou-Samra AB, Jueppner H, Potts JT Jr, Segre GV. Inactivation of pertussis toxin-sensitive guanyl nucleotide-binding proteins increase parathyroid hormone receptors and reverse agonist-induced receptor down-regulation in ROS 17/2.8 cells. Endocrinology 1989;125:2594-2599.
[ccxi] Simonetti RG, Camma C, Fiorello F, Politi F, D'Amico G, Pagliaro L. Hepatocellular carcinoma. A worldwide problem and the major risk factors. Dig Dis Sci 1991;36(7):962-972.
[ccxii] Borzio M, Fargion S, Borzio F, Fracanzani AL, Croce AM, Stroffolini T, Oldani S, Cotichini R, Roncalli M. Impact of large regenerative, low grade and high grade dysplastic nodules in hepatocellular carcinoma development. J Hepatol 2003;39(2):208-214.
[ccxiii] Bosch FX, Ribes J, Cleries R, Diaz M. Epidemiology of hepatocellular carcinoma. Clin Liver Dis 2005;(2):191-211.

[ccxiv] Taylor-Robinson SD, Foster GR, Arora S, Hargreaves S, Thomas HC. Increase in primary liver cancer in the UK, 1979-94. Lancet 1997;350(9085):1142-1143.
[ccxv] McGlynn KA, Tsao L, Hsing AW, Devesa SS, Fraumeni JF Jr. International trends and patterns of primary liver cancer. Int J Cancer 2001;94(2):290-296.
[ccxvi] Bruix J, Sherman M, Llovet JM, Beaugrand M, Lencioni R, Burroughs AK, Christensen E, Pagliaro L, Colombo M, Rodes J; EASL Panel of Experts on HCC. Clinical management of hepatocellular carcinoma. Conclusions of the Barcelona-2000 EASL conference. European Association for the Study of the Liver. J Hepatol 2001;35(3):421-430.
[ccxvii] Daniele B, Bencivenga A, Megna AS, Tinessa V. Alpha-fetoprotein and ultrasonography screening for hepatocellular carcinoma. Gastroenterology 2004 ;127(5 Suppl 1):S108-112.
[ccxviii] de Miguel F, Motellon JL, Hurtado J, Jimenez FJ, Esbrit P. Comparison of two immunoradiometric assays for parathyroid hormone-related protein in the evaluation of cancer patients with and without hypercalcemia. Clin Chim Acta 1998;277(2):171-180.
[ccxix] Minebois-Villegas A, Audran M, Lortholary A, Legrand E, Boux De Casson-Raimbeau F, Jallet P. Performances of two kits for parathyroid hormone-related peptide (PTHrP) assay in the additional study of malignant hypercalcemias Pathol Biol (Paris) 1995;43(9):799-805.
[ccxx] Zolg JW, Langen H. How industry is approaching the search for new diagnostic markers and biomarkers. Mol Cell Proteomics 2004;3(4):345-354.
[ccxxi] Chignard N, Beretta L. Proteomics for hepatocellular carcinoma marker discovery. Gastroenterology 2004;127(5 Suppl 1):S120-5.
[ccxxii] Bruix J, Sala M, Llovet JM. Chemoembolization for hepatocellular carcinoma. Gastroenterology 2004;127(5 Suppl 1):S179-188.
[ccxxiii] Ikeda M, Maeda S, Shibata J, Muta R, Ashihara H, Tanaka M, Fujiyama S, Tomita K. Transcatheter arterial chemotherapy with and without embolization in patients with hepatocellular carcinoma. Oncology 2004;66(1):24-31.
[ccxxiv] Livraghi T, Giorgio A, Marin G, Salmi A, de Sio I, Bolondi L, Pompili M, Brunello F, Lazzaroni S, Torzilli G, et al. Hepatocellular carcinoma and cirrhosis in 746 patients: long-term results of percutaneous ethanol injection. Radiology 1995;197(1):101-108.
[ccxxv] Buscarini L, Buscarini E, Di Stasi M, Vallisa D, Quaretti P, Rocca A. Percutaneous radiofrequency ablation of small hepatocellular carcinoma: long-term results. Eur Radiol 2001;11(6):914-921.
[ccxxvi] Bush DA, Hillebrand DJ, Slater JM, Slater JD. High-dose proton beam radiotherapy of hepatocellular carcinoma: preliminary results of a phase II trial. Gastroenterology 2004;127(5 Suppl 1):S189-193.
[ccxxvii] Geschwind JF, Salem R, Carr BI, Soulen MC, Thurston KG, Goin KA, Van Buskirk M, Roberts CA, Goin JE. Yttrium-90 microspheres for the treatment of hepatocellular carcinoma. Gastroenterology 2004;127(5 Suppl 1):S194-205.
[ccxxviii] Dalhoff K, Dancey J, Astrup L, Skovsgaard T, Hamberg KJ, Lofts FJ, Rosmorduc O, Erlinger S, Bach Hansen J, Steward WP, Skov T, Burcharth F, Evans TR. A phase II study of the vitamin D analogue Seocalcitol in patients with inoperable hepatocellular carcinoma. Br J Cancer 2003;89:252-257.
[ccxxix] Morris DL, Jourdan JL, Finlay I, Gruenberger T, The MP, Pourgholami MH. Hepatic intra-arterial injection of 1,25-dihydroxyvitamin D3 in lipiodol: Pilot study in patients with hepatocellular carcinoma. Int J Oncol 2002;21:901-906.
[ccxxx] Gascon-Barre M, Demers C, Mirshahi A, Neron S, Zalzal S, Nanci A. The normal liver harbors the vitamin D nuclear receptor in nonparenchymal and biliary epithelial cells. Hepatology 2003;37(5):1034-1042.
[ccxxxi] Pourgholami MH, Morris DL. 1,25-Dihydroxyvitamin D3 in lipiodol for the treatment of hepatocellular carcinoma: cellular, animal and clinical studies. J Steroid Biochem Mol Biol 2004;89-90(1-5):513-518.
[ccxxxii] Tovar Sepulveda VA, Falzon M. Regulation of PTH-related protein gene expression by vitamin D in PC-3 prostate cancer cells. Mol Cell Endocrinol 2002;190:115-124.
[ccxxxiii] Amizuka N, Kwan MY, Goltzman D, Ozawa H, White JH. Vitamin D3 differentially regulates parathyroid hormone/parathyroid hormone-related peptide receptor expression in bone and cartilage. J Clin Invest 1999;103(3):373-381.
[ccxxxiv] Lowenfels AB, Maisonneuve P, DiMagno EP, Elitsur Y, Gates LK Jr, Perrault J, Whitcomb DC. Hereditary pancreatitis and the risk of pancreatic cancer. International Hereditary Pancreatitis Study Group. J Natl Cancer Inst 1997;89(6):442-446.

[ccxxxv] Rivera JA, Fernandez-del Castillo C, Pins M, Compton CC, Lewandrowski KB, Rattner DW, Warshaw AL. Pancreatic mucinous ductal ectasia and intraductal papillary neoplasms. A single malignant clinicopathologic entity. Ann Surg 1997 ;225(6):637-644.
[ccxxxvi] Kahl S, Glasbrenner B, Zimmermann S, Malfertheiner P. Endoscopic ultrasound in pancreatic diseases. Dig Dis 2002;20(2):120-126.
[ccxxxvii] Pleskow DK, Berger HJ, Gyves J, Allen E, McLean A, Podolsky DK. Evaluation of a serologic marker, CA19-9, in the diagnosis of pancreatic cancer. Ann Intern Med. 1989;110(9):704-709.
[ccxxxviii] Safi F, Schlosser W, Falkenreck S, Beger HG. CA 19-9 serum course and prognosis of pancreatic cancer. Int J Pancreatol 1996;20(3):155-161.
[ccxxxix] Albert MB, Steinberg WM, Henry JP. Elevated serum levels of tumor marker CA19-9 in acute cholangitis. Dig Dis Sci 1988;33(10):1223-1225.
[ccxl] Bouvet M, Nardin SR, Burton DW, Lee NC, Yang M, Wang X, Baranov E, Behling C, Moossa AR, Hoffman RM, Deftos LJ. Parathyroid hormone-related protein as a novel tumor marker in pancreatic adenocarcinoma. Pancreas 2002;24(3):284-290.
[ccxli] Panzuto F, Severi C, Cannizzaro R, Falconi M, Angeletti S, Pasquali A, Corleto VD, Annibale B, Buonadonna A, Pederzoli P, Delle Fave G. Utility of combined use of plasma levels of chromogranin A and pancreatic polypeptide in the diagnosis of gastrointestinal and pancreatic endocrine tumors. J Endocrinol Invest. 2004;27(1):6-11.
[ccxlii] Peracchi M, Conte D, Gebbia C, Penati C, Pizzinelli S, Arosio M, Corbetta S, Spada A. Plasma chromogranin A in patients with sporadic gastro-entero-pancreatic neuroendocrine tumors or multiple endocrine neoplasia type 1. Eur J Endocrinol. 2003;148(1):39-43.
[ccxliii] Nobels FR, Kwekkeboom DJ, Bouillon R, Lamberts SW. Chromogranin A: its clinical value as marker of neuroendocrine tumours. Eur J Clin Invest. 1998;28(6):431-440.
[ccxliv] Mohr L, Yeung A, Aloman C, Wittrup D, Wands JR. Antibody-directed therapy for human hepatocellular carcinoma. Gastroenterology. 2004;127(5 Suppl 1):S225-231.
[ccxlv] Sato K, Onuma E, Yocum RC, Ogata E. Treatment of malignancy-associated hypercalcemia and cachexia with humanized anti-parathyroid hormone-related protein antibody. Semin Oncol. 2003;30(5 Suppl 16):167-173.
[ccxlvi] Onuma E, Sato K, Saito H, Tsunenari T, Ishii K, Esaki K, Yabuta N, Wakahara Y, Yamada-Okabe H, Ogata E. Generation of a humanized monoclonal antibody against human parathyroid hormone-related protein and its efficacy against humoral hypercalcemia of malignancy. Anticancer Res. 2004;24(5A):2665-2673.
[ccxlvii] Nagy N, Bronckart Y, Camby I, Legendre H, Lahm H, Kaltner H, Hadari Y, Van Ham P, Yeaton P, Pector JC, Zick Y, Salmon I, Danguy A, Kiss R, Gabius HJ. Galectin-8 expression decreases in cancer compared with normal and dysplastic human colon tissue and acts significantly on human colon cancer cell migration as a suppressor. Gut 2002;50(3):392-401.
[ccxlviii] Harris JC, Clarke PA, Awan A, Jankowski J, Watson SA. An antiapoptotic role for gastrin and the gastrin/CCK-2 receptor in Barrett's esophagus. Cancer Res 2004;64(6):1915-1919.
[ccxlix] Brett BT, Smith SC, Bouvier CV, Michaeli D, Hochhauser D, Davidson BR, Kurzawinski TR, Watkinson AF, Van Someren N, Pounder RE, Caplin ME. Phase II study of anti-gastrin-17 antibodies, raised to G17DT, in advanced pancreatic cancer. J Clin Oncol. 2002;20(20):4225-4231.
[ccl] Gilliam AD, Watson SA, Henwood M, McKenzie AJ, Humphreys JE, Elder J, Iftikhar SY, Welch N, Fielding J, Broome P, Michaeli D. A phase II study of G17DT in gastric carcinoma. Eur J Surg Oncol. 2004;30(5):536-543.
[ccli] Bradwell AR, Harvey TC. Control of hypercalcaemia of parathyroid carcinoma by immunisation. Lancet. 1999;353(9150):370-273.
[cclii] Ogata E. Parathyroid hormone-related protein as a potential target of therapy for cancer-associated morbidity. Cancer 2000;88(12 Suppl):2909-29011.

# APPENDICES

## Appendix I

Background information on F423-1 ascites fluid lot P240501

### A. Peptide

The PTHrP immunomimic consisted of the 10 amino terminal amino acids (N-terminal): PTHrP (1-10)-spacer (= N-terminal): Ala-Val-Ser-Glu-His-Gln-Leu-Leu-His-Asp-Ser-Ser-Cys, M.W. 1425.56.

### B. Conjugate

The peptide was conjugated to diphtheria toxoid ("DT") following Aphton's standard laboratory coupling method. EMCS was used as the cross linking agent. The final substitution ratios per $10^5$ molecular weight DT were 9.2. The conjugate was lyophilized.

### C. Immunogen

The conjugate was formulated as water in oil emulsion using montanide ISA 703 as a vehicle in a 70:30 (oil:water) weight/weight ratio (conjugate concentration 1.0 mg/ml). The emulsions were prepared by a hand mixing technique. The emulsion was used to immunize mice.

### D. Immunogenicity/Assays

Female CAF1/J mice were immunized with the emulsion at a dose of 100 μg in 0.1 ml, given intraperitonially. Five mice were immunized with the conjugate. The mice were injected on days 0, 14 and 28 and bled on days 0 and 28.

Serum anti-PTHrP antibody levels were determined in the mouse serum by enzyme-linked-immunosorbent-assay ("ELISA").

A solid phase ELISA was developed to measure anti-PTHrP antibody in the sera. The target antigen for direct binding ELISA was PTHrP (1-40) (Sigma-Aldrich, Inc, UK).

## E. Hybridoma

Monoclonal antibody against PTHrP would be suitable for diagnostic and therapeutic applications. In order to obtain a monoclonal antibody specific against the PTHrP (1-10) peptide, a cell fusion was performed.

P3/NS-1 (non-secreting myeloma, mouse) cells were fused with spleen cells from a CAF1/J mouse that was immunized with PTHrP(1-10)-spacer-DTc conjugate. Four days prior to cell fusion, the mice received an aqueous boost of 0.1 mg conjugate/mouse. The method used for the cell fusion and hybridoma production was similar to the protocol in Selected Methods in Immunology[cclii]. Clones producing anti-PTHrP antibody were selected by ELISA, with PTHrP (1-40) as the target antigen and inhibited with PTHrP (1-10) epitope peptide. Alkaline phosphatase conjugated rabbit anti-mouse IgG+A+M (H+L) antibody was used as the developing reagent. Four final hybridoma lines were selected and named F423-1, F423-2, F423-3 and F423-4. The isotype of lines F423-1 to F423-3 were IgG1 and F423-4 was IgG2b. Line F423-1 gave the highest binding by ELISA and the highest inhibition by PTHrP (1-10) epitope peptide (94%); while the 3 other lines gave comparable values, with line F423-4 being the lowest antibody producer.

F423-1, anti-PTHrP (1-10) Ascites in Balb/CJ mice Lot P240501

**PURPOSE**: to produce F423-1 mouse ascites fluid, anti-PTHrP(1-10) IgG1 MAb in 6 Balb/CJ female mice primed with Pristane.

**MATERIALS:**

- Fusion # 423-1 (anti-PTHrP (1-10)-DTc) MAb cell line
- 6 Balb/CJ female mice, retired breeders from Jackson Labs (Davis, CA, USA) (date of birth: 05/00, received 02/07/01)
- Pristane (Sigma)

- Sterile PBS.
- Sorvall® RC-5B refrigerated superspeed centrifuge (Dupont, DE, USA), with Sorvall® SS-34 rotor, angular velocity setting of 10 revolutions/minute (RPM) x 1000. Aphton equipment # EQ0038.
- Glass syringe filter (Gelman Sciences, MI, USA)
- Whatman® filter papers (Whatman Inc, NJ, USA): # 1, # 3 and # 5 (2.5 μm)
- 0.22 μm cellulose acetate, 150 ml Tube Top filter (Corning, NY, USA).

**METHOD:**

*14-Mar-01 (Day –19), Priming mice with Pristane*

1. Six mice were injected with 0.5 ml/mouse of Pristane using a 21 & 23G needle by first going under subcutaneous tissue for approximately half a centimeter and then intraperitoneally to avoid oil leakage.

*2-Apr-01 (Day 0), Injection 1 of mice with fusion cells*

2. Cells were harvested from T-flasks (T-75 & T-25) into a 50 ml centrifuge tube (47 ml cells).
3. Cells were centrifuged at 400 g for 10 minutes at room temperature.
4. Supernatant was collected and saved. The centrifuge tube was gently tapped to break the cell pellet to which PBS was added and then mixed well.
5. Viable cells were counted using a haemocytometer.
6. All six mice were injected intraperitoneally with 4 x $10^6$ cells/mouse.

*Ascites tap on different days*

Mice were tapped for ascites, which was spun with high speed centrifuge at 10000 RPM for 30 minutes, collected, and stored frozen until ready to use.

*25-May-01, ascites pool/filtration*

1. All the ascites was thawed.
2. Fatty clots were removed and the fluid was pooled.

3. In a laminar flow-hood, the fluid was filtered using a Glass Acrodisc, and with Whatman® filter papers in an Erlenmeyer flask. The filtration process was repeated with decreasing membrane size filters until most of the fat was removed. For the final sterile filtration, a 150 ml bottle top 0.22 μm CA filter with filter paper was used.

4. The final 38 ml (19 ml x 2 vials; 0.6 ml x 1 vial for assays) lot was named as P240501, and stored -20°C in a chest freezer (later moved into the lab REVCO at -70°C).

# Appendix II

## Anti-PTHrP

A novel monoclonal antibody specific to PTHrP was raised in a mouse to the first 10 amino terminal amino acids of human PTHrP (amino acids 1-10) (kindly provided by Aphton Corporation, Woodland, USA). This antibody is specific to human and rat PTHrP, and does not show any cross-reactivity with PTH (Appendix I).
The specificity of staining of the antibody was initially established on the following cases: regenerating liver, where there was cytoplasmic staining of the proliferating bile ductules, as previously described[cclii] (Figure 1a); and cases of breast carcinoma, where there was cytoplasmic staining of tumour cells, which is also well documented in the majority of tumours.[cclii] Negative control sections were obtained by omitting the primary antibody. The specificity of positive staining was determined by pre-absorbing the same concentration of antibody with an excess concentration of the immunizing peptide overnight at 4°C. When the antibody-antigen solution was applied to serial tissue sections adjacent to the antibody-treated sections, staining was completely abolished (Figure 1b).

After optimisation experiments on control tissue, a dilution of 1 in 1000 in tris buffered saline (TBS) was selected for use on the test tissues. Antigenic unmasking was carried out by pressure cooking the slides at 115°C in 0.1 M citrate buffer, pH 6.0, for 90 seconds.

**Anti-PTH1R**

PTH1R expression was detected using a commercially available mouse monoclonal anti-PTH1R antibody (Clone 3D1.1) (Labvision Corporation, USA), raised against an epitope located in amino acids 146-169 of the human PTH/PTHrP receptor (PTH1R). PTH1R has previously been shown to be expressed in human breast adenocarcinoma,[cclii] and therefore a well-characterised case of human breast adenocarcinoma was selected for use as a control in which cytoplasmic staining was seen. A case of regenerating liver was also tested with the antibody, and positive staining was found in the bile ductules (Figure 1c).

After optimization experiments on the control tissue, a primary antibody dilution of 1 in 50 in TBS was used in subsequent experiments. Antigenic unmasking was performed by microwaving the slides in 0.1 M citrate buffer, pH 6.0, for 20 minutes.

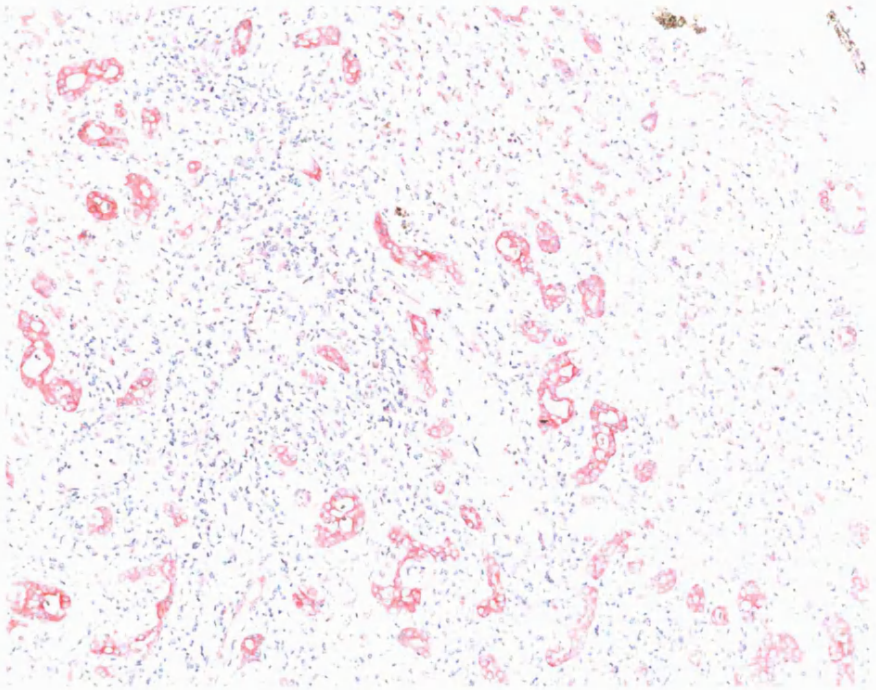


Figure 1 (a)

Figure 1 Immunohistochemistry of PTHrP (1-10) (a) and PTH1R (c) on sections of regenerating liver.

Positive cytoplasmic staining of the bile ductules appears as a red precipitate. Staining for PTHrP (1-10) is abolished by preabsorption of the antibody with peptide (Figure b). Images photographed at magnification x 100.

Figure 1 (b)

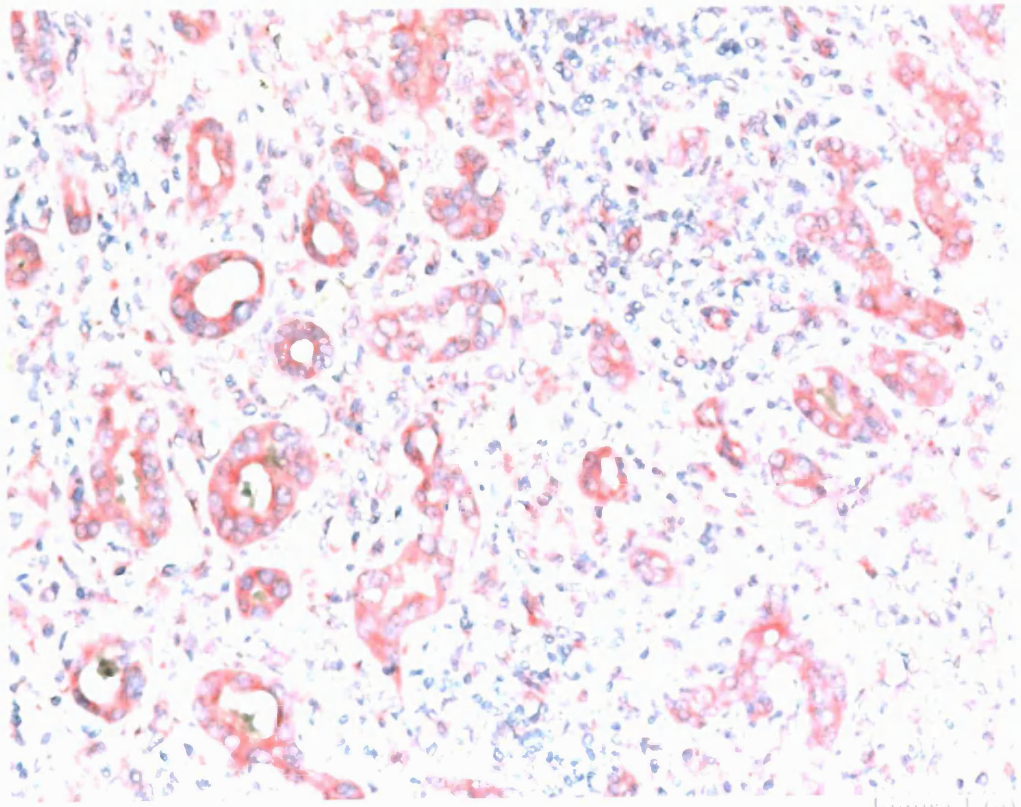


Figure 1 (c)

## Appendix III

### The APAAP method

Endogenous alkaline phosphatase activity was blocked by incubation in 15% acetic acid for 20 minutes. To unmask antigenic binding sites, slides were then heated in 0.1 M citrate buffer, pH 6.0, before being allowed to cool to room temperature. In order to block non-specific binding of the secondary antibody, the slides were incubated in 10% normal rabbit serum (DakoCytomation, Ely, UK) in tris-buffered saline (TBS) for 20 minutes. The slides were then incubated with the primary antibody for 1 hour at room temperature. Rabbit anti-mouse immunoglobulin (DakoCytomation, Ely, UK) at a dilution of 1 in 50 in TBS, with 1 in 25 dilution of normal human serum, was applied for 30 minutes and then collected. The tertiary antibody, mouse monoclonal alkaline phosphatase anti-alkaline phosphatase (APAAP) at a dilution of 1 in 40 in TBS was applied for 30 minutes and then collected. These antibodies were then sequentially reapplied for 20 minutes each. During each step, the slides were rinsed in TBS for 5 minutes. Positive staining was visualised using Fast Red (Sigma) as chromogen, incubating at room temperature for 10–20 minutes. Nuclei were counterstained with Meyer's haematoxylin, and the slides were air dried and mounted in Loctite UV (Loctite, UK) adhesive.

### The ABC method

The slides were incubated with 0.5% hydrogen peroxide in methanol for 10 minutes. For the detection of the Ki67 antigen, antigenic unmasking was carried out by pressure cooking the slides at 115°C in 0.1 M citrate buffer, pH 6.0, for 135 seconds. In order to block non-specific binding of the secondary antibody, the slides were incubated in 10% normal goat serum (Dako) in TBS for 20 minutes, and then incubated with the primary antibody for 1 hour at room temperature. After rinsing with TBS, the sections were incubated for 30 minutes with biotinylated affinity-purified goat antibody to mouse/rabbit immunoglobulins (Dako) (diluted to 1 in 100 in TBS, with 1 in 25 dilution of normal human serum). After a further 5 minute rinse in TBS, the sections were incubated with streptavidin (Dako) (1 in 100 dilution in TBS) and biotinylated horseradish peroxidase (Dako) (1 in 100 dilution in TBS,

prepared 30 minutes before application) for 30 minutes. After a final rinse in TBS, the sections were incubated with the prepared chromogenic substrate solution for peroxidase, DAB (Vector Laboratories Ltd, UK), for 10 minutes. Slides were rinsed in distilled water and counterstained with Meyer's haematoxylin. Slides were then dehydrated through alcohol and xylene, and mounted.